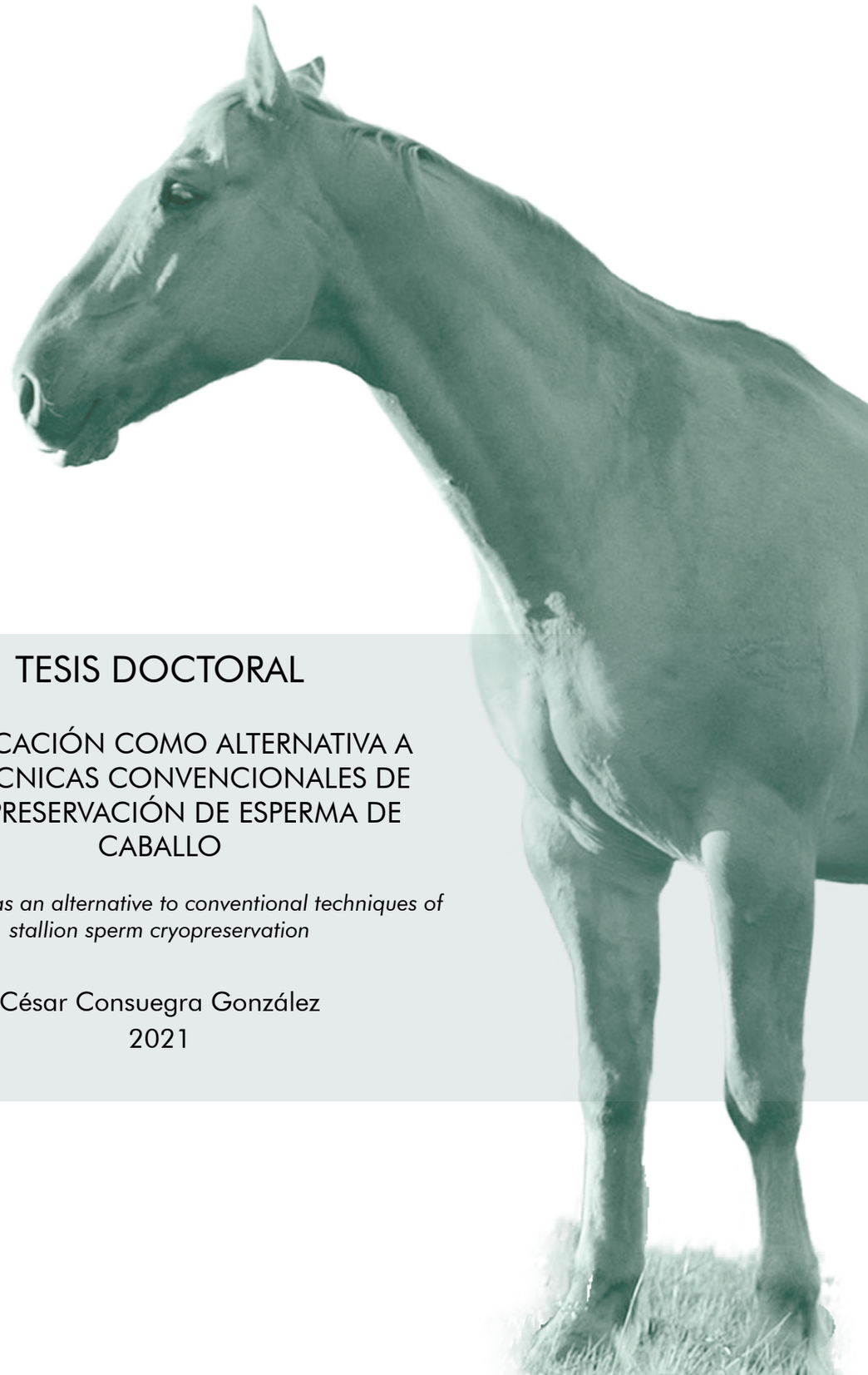
César Consuegra González - Vitrificación como alternativa a las técnicas convencionales de criopreservación de esperma de caballo



TESIS DOCTORAL

VITRIFICACIÓN COMO ALTERNATIVA A LAS TÉCNICAS CONVENCIONALES DE CRIOPRESERVACIÓN DE ESPERMA DE CABALLO

Vitrification as an alternative to conventional techniques of stallion sperm cryopreservation

César Consuegra González

2021

UNIVERSIDAD D CÓRDOBA

TITULO: *Vitrification as an alternative to conventional techniques of stallion sperm cryopreservation*

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UNIVERSIDAD DE CÓRDOBA

Programa de Doctorado Biociencias y Ciencias Agroalimentarias

VITRIFICACIÓN COMO ALTERNATIVA A LAS TÉCNICAS CONVENCIONALES DE CRIOPRESERVACIÓN DE ESPERMA DE CABALLO

Vitrification as an alternative to conventional techniques of stallion sperm cryopreservation

Memoria para optar al grado de Doctor presentada por:

CÉSAR CONSUEGRA GONZÁLEZ

Bajo la dirección de los directores:

Manuel Hidalgo Prieto

Jesús M. Dorado Martín

Francisco Crespo Castejón

Córdoba, febrero 2021

FINANCIACIÓN

El doctorando ha disfrutado de una ayuda para la Formación de Profesorado Universitario (FPU16/06745), concedida por el Ministerio de Educación, Cultura y Deporte, en el marco del Plan Estatal de Investigación Científica y Técnica y de Innovación 2013-2016.

La estancia realizada durante tres meses en el Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (INIA) de Madrid, fue financiada por las ayudas complementarias de movilidad del Ministerio de Educación y Formación Profesional, convocatoria 2018, destinadas a beneficiarios del Programa de Formación del Profesorado Universitario (FPU).

La estancia realizada para la obtención de la Mención Internacional de la Tesis Doctoral, con una duración de tres meses en la Universidad de Linköping (Suecia), fue financiada por las ayudas complementarias del Ministerio de Educación y Formación Profesional, convocatoria 2020, destinadas a beneficiarios del Programa de Formación del Profesorado Universitario (FPU).

El proyecto AGL-2013-42726-R titulado “Optimización de la técnica de vitrificación de gametos y embriones equinos” concedido por la Dirección General de Investigación Científica y Técnica del Ministerio de Economía y Competitividad, ha financiado la parte experimental del presente trabajo de Tesis Doctoral.

TÍTULO DE LA TESIS:

Vitrificación como alternativa a las técnicas convencionales de criopreservación de esperma de caballo.

DOCTORANDO: César Consuegra González

INFORME RAZONADO DEL/DE LOS DIRECTOR/ES DE LA TESIS:

D. Manuel Hidalgo Prieto y D. Jesús M. Dorado Martín, profesores titulares del Departamento de Medicina y Cirugía Animal de la Facultad de Veterinaria de la Universidad de Córdoba, y D. Francisco Crespo Castejón, Teniente Coronel jefe del Centro Militar de Cría Caballar de Ávila y Profesor asociado del Departamento de Medicina y Cirugía Animal de la Facultad de Veterinaria la Universidad Complutense de Madrid.

INFORMAN:

Que el trabajo de Tesis titulado "Vitrificación como alternativa a las técnicas convencionales de criopreservación de esperma de caballo" ha sido realizado por D. César Consuegra González. El objetivo principal de la tesis fue desarrollar la técnica de la vitrificación para la criopreservación de esperma de caballo, como alternativa a las técnicas convencionales de criopreservación.

Parte de los resultados obtenidos durante el desarrollo de la presente Tesis Doctoral han sido publicados como siete artículos científicos en tres revistas diferentes indexadas en el JCR, siendo ésta elaborada por compendio de publicaciones:

Tesis Doctoral por compendio de publicaciones:

C Consuegra, F Crespo, M Bottrel, I Ortiz, J Dorado, M Diaz-Jimenez, B Pereira, M Hidalgo (2018). Stallion sperm freezing with sucrose extenders: A strategy to avoid permeable cryoprotectants. Animal Reproduction Science.191.85-91.

M Hidalgo, **C Consuegra**, J Dorado, M Diaz-Jimenez, I Ortiz, B Pereira, R Sánchez, F Crespo (2018). Concentrations of non-permeable cryoprotectants and equilibration temperatures are key factors for stallion sperm vitrification success. Animal Reproduction Science. 196. 91-98.

C Consuegra, F Crespo, J Dorado, I Ortiz, M Diaz-Jimenez, B Pereira, M Hidalgo (2018). Comparison of different sucrose based extenders for stallion sperm vitrification in straws. Reproduction in Domestic Animals. 53. 57-59.

C Consuegra, F Crespo, J Dorado, M Diaz-Jimenez, B Pereira, I Ortiz, M Hidalgo (2019). Vitrification of stallion sperm using 0.25 mL straws: Effect of volume, concentration and carbohydrates (sucrose/trehalose/raffinose).Animal Reproduction Science. 206. 69-77.

C Consuegra, F Crespo, J Dorado, M Diaz-Jimenez, B Pereira, M Hidalgo (2019). Low-density lipoproteins and milk serum proteins improve the quality of stallion sperm after vitrification in straws. Reproduction in Domestic Animals. 54. s4. 86-89.

C Consuegra, F Crespo, J Dorado, M Diaz-Jimenez, B Pereira, I Ortiz, R Arenas, J Morrell, M Hidalgo (2019). Vitrification of large volumes of stallion sperm in comparison to spheres and conventional freezing: effect of warming procedures and sperm selection. Journal of Equine Veterinary Science. 83. 102680.

C Consuegra, F Crespo, J Dorado, M Diaz-Jimenez, B Pereira, M J Sánchez-Calabuig, P Beltrán-Breña, S Pérez-Cerezales, D Rizos, M Hidalgo (2020). Fertilizing capacity of vitrified stallion sperm assessed utilizing heterologous IVF after different semen warming procedures. Animal Reproduction Science. 223. 106627.

En referencia al Plan de Formación para el Doctorado en el Programa de Biociencias y Ciencias Agroalimentarias, el doctorando ha realizado satisfactoriamente las actividades obligatorias propuestas, así como distintas actividades optativas por curso, tal y como se recoge en el documento de actividades formativas que acompaña esta tesis.

Con objeto de ampliar su formación y profundizar en el estudio de la vitrificación de esperma, el doctorando realizó una estancia de tres meses en el Departamento de Reproducción Animal en el Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (INIA) de Madrid. Asimismo, para optar a la Mención Internacional, el doctorando realizó una estancia de tres meses en el Departamento de Ciencias Biomédicas y Clínicas de la Universidad de Linköping (Suecia).

La presente Tesis Doctoral ha sido revisada, reuniendo a nuestro juicio todos los requisitos necesarios para su lectura y defensa por compendio de publicaciones, así como para obtener la Mención Internacional.

Y para que conste, en cumplimiento de las disposiciones vigentes, se autoriza la lectura y defensa de la tesis doctoral.

Córdoba, 24 de febrero de 2021

Firma de los directores

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Fdo.: Francisco Crespo Castejón

INTERNATIONAL DOCTORS REPORT
DOCTORAL THESIS

REFEREE REPORT ON THE PhD TESIS PRESENTED IN THE UNIVERSITY OF CÓRDOBA (SPAIN) BY **CÉSAR CONSUEGRA GONZÁLEZ**.

TITLE OF THE THESIS: Vitrification as an alternative to conventional techniques of stallion sperm cryopreservation

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This thesis meets the requirements for presentation as an oral dissertation: ● YES ○ NO

RATING

Originality:	● Outstanding	○ Excellent	○ Very Good	○ Good	○ Sound	○ Defficient
Scientific/ technical merit:	● Outstanding	○ Excellent	○ Very Good	○ Good	○ Sound	○ Defficient
Planning/ methodology:	● Outstanding	○ Excellent	○ Very Good	○ Good	○ Sound	○ Defficient

COMMENTS (Please use additional sheets, if necessary):

The semen cryopreservation is a key biotechnological strategy used to preserve and protect genetic resources of certain biologically or scientifically valuable males. Nevertheless, it is a harmful process and induces many unfavorable changes in the spermatozoa. During this process, spermatozoa are exposed simultaneously to damage caused by thermal shock, freezing and thawing, which consequently cause osmotic changes in the cell membrane and reorganization of its lipids and proteins. Therefore, cryopreservation requires protection of intracellular structures and biomolecules, and hence requires protective agents that are able to pass the cellular membrane. Cryopreservation of stallion sperm has been traditionally performed by slow freezing methods, including the use of different permeable cryoprotectants(CPAs). However,

some permeable CPAs are toxic for the sperm cells and reduce the success of cryopreservation. Therefore, there is need to still search for optimal freezing agents such as non-permeable agents and freezing protocols to ensure the highest possible quality of thawed sperm and to maintain their fertilizing capacity. Kinetic vitrification is a procedure that was developed as an alternative to conventional freezing for cryopreservation of sperm. There is a rapid cooling rate to obtain a glass-like solidification state of sperm cells in this procedure.
The presented work describes the development of the technique of stallion semen vitrification taking into account every important factor for success. In this thesis it was found that vitrification of stallion sperm using non-permeable CPAs can be used as an alternative to conventional sperm freezing.

1. Format
The thesis is clear and well organized. The title and the abstract reflect the contents and the introduction clearly states the problem being investigated and provides an adequate background. The present thesis consists: summary, introduction, objectives, chapters with papers, conclusions, references, quality indicators and other scientific contributions derived directly from the Doctoral Thesis.
Despite the lack of a general discussion, each paper contains discussion section that strongly support the results collected from the different experiments carried out. It would be very difficult to construct one compact discussion for each experiment performed in these seven papers.

2. Methodology
The materials & methods section in each paper is described in details allowing repeat such experiments. The methods of semen assessment was chosen adequately. Techniques used for sperm quality assessment includes: CASA system for sperm motility parameters (Sperm Class Analyzer), Vital Test for plasma membrane integrity, fluorescein conjugated lectin PNA and propidium iodide for acrosome membrane integrity, and also Sperm Chromatin Structure Assay for sperm DNA integrity. Moreover in the last publication heterologous in vitro fertilization test was performed.
Statistical procedures were adequate for data analysis from each experiment. There were used general linear model, ANOVA, linear regression and Duncan or Tuckey tests.

3. Publications
This thesis includes 5 thematic chapters, which each consists very valuable results. Chapter 1st, 2nd, 3rd and 5th consists of one publication and chapter 4th is divided into 3 subsections contain 3 publications. The division was very logical and corresponding to five presented objectives.
In the first paper very important results are presented, showing that stallion sperm can be frozen in the absence of permeable cryoprotectants using 100 mM of sucrose and 1% of BSA. So far no studies have been conducted combining these additives as non-permeable agents in horses.
In the second article the protocol of stallion semen vitrification in spheres with 20 mM of sucrose and 1% BSA has revealed better sperm parameters than sperm freezing with glycerol.
The third publication confirmed above results. Vitrification in spheres of stallion sperm obtained better semen quality than conventional freezing or vitrification in 0.5 mL straws.

In the next three publications included in chapter 4, the protocol of stallion sperm vitrification using 0.25 mL straws was developed. In fourth publication, which is short communication, the result from first publication were confirmed. Extender with 100 mM of sucrose obtained the best results. However, in the next experiments other sugar was better. It was observed that the use of 0.25 mL straws filled with 100 uL and 100 mln of spermatozoa/mL using 100 mM trehalose and 1% BSA diluent was the best method for vitrification semen of this species (fifth publication). Furthermore, applying 0.25% LDL and 1% Pronexcell improved the sperm quality after vitrification (sixth publication). The last publication concerns the subject of the optimal method of warming of vitrified semen samples and was completed with an important achievement performing heterologous in vitro fertilization. Vitrified doses of stallion semen can be warmed at 60 C degree for 5s. Furthermore, vitrified sperm are able to penetrate cattle oocytes and have a similar fertilizing capacity as conventionally frozen stallion semen.

4. Scientific value

The journals where the manuscripts have been published are recognized as having high scientific reputation. Animal Reproduction Science and Reproduction in Domestic Animals are leaders on the field of animal reproduction with high impact and citation indices. Journal of Equine Veterinary Science is an international journal designed for the practicing equine veterinarian, equine researcher and other equine health care specialist with high range. Therefore, all journals confirm a very high scientific relevance of the present thesis. Additional abstracts presented during international meetings also confirm the significant scientific value of presented work.

I would like to emphasize once again that this thesis consists of seven published articles, which confirms remarkable commitment to scientific work of PhD student.

For all the evidence provided, I can strongly recommend the PhD candidate, César Consuegra González, for the PhD degree.

Wrocław,

DATE: 22.02.2021 **SIGNATURE:** Agnieszka Partyka

INTERNATIONAL DOCTORS REPORT
DOCTORAL THESIS

REFEREE REPORT ON THE PhD TESIS PRESENTED IN THE UNIVERSITY OF CÓRDOBA (SPAIN) BY **CÉSAR CONSUEGRA GONZÁLEZ**.

TITLE OF THE THESIS: Vitrification as an alternative to conventional techniques of stallion sperm cryopreservation

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This thesis meets the requirements for presentation as an oral dissertation: ☒ YES ☐ NO

RATING

Originality:	☐ Outstanding	☒ Excellent	☐ Very Good	☐ Good	☐ Sound	☐ Defficient
Scientific/ technical merit:	☒ Outstanding	☐ Excellent	☐ Very Good	☐ Good	☐ Sound	☐ Defficient
Planning/ methodology:	☒ Outstanding	☐ Excellent	☐ Very Good	☐ Good	☐ Sound	☐ Defficient

COMMENTS (Please use additional sheets, if necessary):

This Thesis entitled "Vitrification as an alternative to conventional techniques of stallion sperm cryopreservation" presented by César Consegura Gonzalez is very well presented, structured and written. This PhD Thesis includes relevant information regarding the development and optimization of stallion sperm freezing and vitrification techniques. The aim of this Thesis was to develop vitrification as alternative methods for stallion sperm cryopreservation. The Thesis starts with a very clear abstract, in Spanish and English. It is followed by a thorough Introduction on the subject, and by five very well defined and important Objectives. To each Objective a Thesis chapter is dedicated, made up by published papers. Overall, this Thesis includes 7 papers, in 6 of which the candidate is the first authors, published in International peer-reviewed journals. Each experiment was planned and described with care, using a proper scientific approach, appropiate experimental designs and up-to date methods for

the evaluation of results (motility analyser, fluorescence microscopy for plasma membrane, acrosome and DNA integrity, heterologous zona binding and IVF assay). Each aim of the studies was reached. In Chapter 4, the vitrification method developed in this Thesis gave overall better results than the convetional method for stallion semen preservation to which it was compared. In Chapter 5, sperm cells vitrified using the method here described had the capacity to penetrate cattle oocytes, leading to pronuclear formation and hybrid embryo cleavage. The objective conclusion of this Thesis was that, according to the results obtained, vitrification of stallion sperm using non-permeable CPAs can be used as an alternative method to conventional freezing.
The topic of this PhD thesis is very interesting, innovative for stallion semen cryopreservation, and thus adequate for a PhD project.

This Thesis shows that César Consegura Gonzalez has acquired deep knowledge of the subject and valuable research skills, such as those developed during the field and laboratory experiences, the analysis of data and the discussion of the results.

In conclusion, this Doctoral thesis is worth for César Consegura Gonzalez to be awarded the degree of European Doctorate.

DATE:17/02/2021 **SIGNATURE:** Alejandro Peña

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PROGRAMA ESTATAL DE PROMOCIÓN DEL TALENTO Y SU EMPLEABILIDAD
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Nombre del Organismo receptor / *Name of the receiving institution*:

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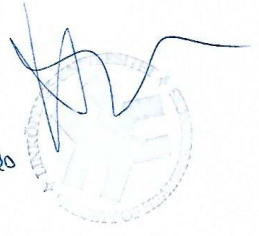

Linköping, 30th November 2020

AGRADECIMIENTOS

AGRADECIMIENTOS

En primer lugar quiero dar las gracias a mi director Manuel Hidalgo Prieto. Gracias por darme la oportunidad de llevar a cabo mi Tesis Doctoral, y poner a mi disposición todos los medios necesarios. Gracias por guiarme y orientarme en estos años, por estar constantemente informándome de oportunidades para aprender y seguir formándome.

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Asimismo, gracias a mi director Francisco Crespo Castejón por todas las facilidades que me ha dado para realizar mis estudios, y acogerme en Ávila siempre que lo he necesitado.

Muchas gracias a mis compañeras de laboratorio, Mari, Blasi e Isa. Gracias por todos los momentos compartidos juntos, dentro y fuera de la Universidad. Por todo lo que me habéis enseñado, por estar día a día juntos sacando todo para adelante. Pocas personas pueden comprender tan bien como vosotras lo que he vivido durante estos años. Habéis sido una influencia muy importante en mi vida. Sin duda, haberos conocido ha sido de lo mejor que me ha pasado en estos últimos años.

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Al Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria de Madrid, en el que me sentí desde el primer momento como uno más. Por ampliar mis conocimientos y darme a conocer otras vías para desarrollarme profesionalmente. Agradecer en primer lugar a Dimitrios Rizos, que me acogió en su grupo y en su laboratorio, por ayudarme a perfeccionar mi trabajo en el laboratorio. Asimismo, quiero dar las gracias a Julián Santiago-Moreno, María Jesús Sánchez Calabuig, Paula Beltrán, y Serafín Pérez Cerezales.

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Gracias a toda mi familia, a mis abuelos, mis tíos y primos por su apoyo en todo momento. A mi madre Gloria, porque nadie me conoce mejor que tú y sabes cuándo necesito tu ayuda y cariño, pero también cuando necesito estar solo. A mi padre, Pepe, porque somos iguales, y aunque a veces "choquemos", siempre estás ahí para lo que he necesitado, para darme todo lo que fueras capaz. A mis hermanos, Pedro y Javier, por cuidarme y preocuparos por mí, por alegrarme el día cuando regresaba a Úbeda. Porque, aunque a veces no lo demuestre, sois las personas a las que más quiero y más importantes de mi vida.

A mis amigos, por los buenos momentos que hemos vivido en todos estos años, en especial durante esta última época, porque esas quedadas y conversaciones han sido imprescindibles para desconectar. Especialmente, quiero dar las gracias a Ricardo, Andrés, Alberto, Paco, Pablo, Carlos, Claudia y Nuria, amigos de Úbeda de toda la vida, o buena parte de ella. No me quiero olvidar de los baezanos, que, si bien nos conocemos de hace menos tiempo, estáis tan presentes como los demás; muchas gracias Antonio, Vicky y Miriam.

ABREVIATURAS

AIS	Acrosome integrity of sperm
ALH	Amplitude of lateral head displacement
AO	Acridine orange
BCF	Beat cross frequency
BSA	Bovine serum albumen
CASA	Computer-assisted sperm analysis
CPA	Cryoprotector
FITC - PNA	Peanut agglutinin-fluorescein isothiocyanate
IVF	In vitro fertilization
hpi	Hours post-insemination
AI	Artificial insemination
IMS	Integrity of the membrane of sperm
LDL	Low-density lipoproteins
LIN	Linearity
MW	Molecular weight
LN_2	Liquid nitrogen
PI	Propidium iodide
PM	Progressive motility
SCSA	Sperm chromatin structure assay
SLC	Single layer centrifugation
STR	Straightness
TM	Total motility
VAP	Average path velocity
VCL	Curvilinear line velocity
VSL	Straight line velocity
WOB	Wobble

ÍNDICE

ÍNDICE

RESUMEN

RESUMEN

El desarrollo de la criopreservación de esperma ha sido crucial para la conservación de recursos genéticos animales y la mejora de la eficiencia reproductiva de diferentes especies. La vitrificación cinética del esperma es una técnica que se ha desarrollado recientemente como alternativa a los métodos de criopreservación convencional. Se basa en la inmersión directa en nitrógeno líquido de una suspensión de espermatozoides sin crioprotectores (CPAs) permeables, siendo una de sus principales ventajas la reducción del daño celular debido a la ausencia de cristales de hielo intracelulares. Por tanto, en la presente Tesis Doctoral se han desarrollado distintos protocolos de vitrificación espermática en la especie equina, como método alternativo de criopreservación.

En el Capítulo 1, se evaluó el empleo de distintas concentraciones de sacarosa como alternativa a los crioprotectores permeables para la congelación de esperma de caballo. La adición de 100 mM de sacarosa y 1% de albúmina sérica bovina (BSA) obtuvo valores mayores para la mayoría de los parámetros cinéticos espermáticos evaluados ($P < 0,001$), mostrando un porcentaje de espermatozoides con membrana acrosómica dañada inferior en comparación con el diluyente con glicerol ($P < 0,05$).

En el Capítulo 2, se evaluó el efecto de los CPAs no permeables y de la temperatura del periodo de equilibrado sobre la calidad de los espermatozoides tras la vitrificación en esferas. La refrigeración a 5ºC y el empleo de una adecuada concentración de CPAs no permeables (20 mM de sacarosa + 1% de BSA) fueron factores clave para la vitrificación de esperma de caballo.

El Capítulo 3 tuvo como objetivo la vitrificación de grandes volúmenes de esperma de caballo, empleando pajuelas de 0,5 mL, y la evaluación de diferentes procedimientos de calentamiento (42ºC / 20s; 60ºC / 15s) y de la centrifugación en una sola capa de coloide (SLC). Los procedimientos de calentamiento de las muestras vitrificadas no mejoraron la calidad de los espermatozoides. Sin embargo la técnica SLC podría ser una estrategia para mejorar la calidad de las muestras vitrificadas en grandes volúmenes, ya que tras esta se obtuvieron valores más altos de movimiento espermático e integridad de la membrana plasmática. En cualquier caso, la calidad de las dosis vitrificadas en pajuelas de 0,5 mL fue inferior a la obtenida tras la vitrificación en esferas.

El Capítulo 4 fue diseñado con el objetivo de optimizar la técnica de vitrificación de esperma de caballo, empleando pajuelas. Así, se observó que pajuelas de 0,25 mL con un volumen de esperma de 100 µl, a una concentración de 100 x 10^6 espermatozoides/mL y utilizando un diluyente que contenía 100 mM de trehalosa y 1% de BSA resultó ser el método óptimo para la vitrificación de esperma de caballo. Este método de vitrificación obtuvo valores mayores en comparación con la congelación convencional para el movimiento progresivo (48,2 ± 2,3% vs. 37,3 ± 2,2%), integridad de la membrana plasmática (82,8 ± 1,5% vs. 74,1 ± 1,9%) y del acrosoma (50,2 ± 1,2% vs. 43,1 ± 1,4%), así como menor fragmentación del ADN (6,4 ± 0,7% vs. 8,2 ± 0,3%). Asimismo, la suplementación del diluyente de vitrificación con 0,25% de lipoproteínas de baja densidad (LDL) y 1% de Pronexcell, mejoró la calidad de los espermatozoides tras la vitrificación.

En el Capítulo 5, se valoró la capacidad fecundante de los espermatozoides de caballo congelados o vitrificados. En primer lugar, se demostró que el calentamiento de pajuelas vitrificadas en un baño de agua a 60ºC/5s puede ser empleado como alternativa al método convencional mediante inmersión en diluyente a 43ºC. Posteriormente, se evaluó la capacidad fecundante de los espermatozoides vitrificados y congelados mediante la técnica de la fecundación in vitro (FIV) heteróloga, utilizando ovocitos de bovino. La vitrificación resultó en valores mayores ($P < 0,05$) que la congelación para el número de espermatozoides unidos a los ovocitos (1,36 ± 0,3 y 0,69 ± 0,2, respectivamente). No hubo diferencias entre los espermatozoides congelados o vitrificados en la formación de los pronúcleos (26 h después de la inseminación - hpi; 14,08 ± 4,2% y 22,78 ± 4,8%, respectivamente) o la tasa de segmentación de embriones (32,77 ± 4,3% y 39,66 ± 4,6%, respectivamente).

En conclusión, de acuerdo con los resultados obtenidos en esta Tesis Doctoral, la vitrificación de esperma de caballo empleando crioprotectores no permeables, puede ser una técnica alternativa a la congelación convencional de esperma.

SUMMARY

SUMMARY

The development of cryopreservation has been crucial for the maintenance of animal genetic resources and the improvement of reproductive efficiency. Kinetic sperm vitrification has been developed mainly as an alternative to conventional freezing. It is based on the direct immersion in liquid nitrogen (LN_2) of a sperm suspension without permeable cryoprotectants (CPAs), being one of its main advantages the reduction of cellular damage due to the absence of intracellular ice crystals. Therefore, in the present Doctoral Thesis, different protocols of sperm vitrification have been developed in the equine species, as an alternative method of cryopreservation.

In Chapter 1, different concentrations of sucrose were evaluated as an alternative to permeable CPAs for stallion sperm freezing. The addition of 100 mM of sucrose and 1% of bovine serum albumen (BSA) obtained higher values for most of the kinetic sperm parameters evaluated ($P<0.001$), showing a lower percentage of spermatozoa with damaged acrosome membrane compared to the extender with glycerol ($P<0.05$).

In Chapter 2, the effect of non-permeable CPAs and equilibration temperature on the quality of the spermatozoa after vitrification in spheres was evaluated. Sperm refrigeration at 5ºC and the use of a proper concentration of non-permeable CPAs (20 mM sucrose + 1% BSA) were key factors for stallion sperm vitrification.

The objective of Chapter 3 was to vitrify large volumes of stallion sperm using 0.5 mL straws, and the assessment of different warming procedures (42°C / 20s; 60°C / 15s) and single layer centrifugation (SLC). The warming procedures of vitrified samples did not improve the quality of the sperm. However, SLC technique could be a strategy to enhance the quality of the samples of sperm vitrified in large volumes, since higher values of sperm motility and plasma membrane integrity were obtained with this technique. Nevertheless, the sperm quality obtained after vitrification in 0.5 mL straws was lower than after vitrification in spheres.

Chapter 4 was designed with the objective of developing the vitrification technique for stallion sperm using 0.25 mL straws. Thus, it was observed that the use of 0.25 mL straws filled with 100 µl and 100 x 10^6 spermatozoa/mL using an extender containing 100 mM trehalose and 1% BSA was the optimal method of stallion sperm vitrification. This vitrification method obtained higher values in comparison to conventional freezing for progressive motility (48.2 ± 2.3% vs. 37.3 ± 2.2%), integrity of plasma (82.8 ± 1.5% vs. 74.1 ± 1.9%) and acrosome membrane (50.2 ± 1.2% vs. 43.1 ± 1.4%), as well as less DNA fragmentation (6.4 ± 0.7% vs. 8.2 ± 0.3%). Likewise, the supplementation of the vitrification extender with 0.25% low-density lipoprotein (LDL) and 1% Pronexcell improve the quality of the sperm after vitrification.

In Chapter 5, the objective was to assess the fertilizing capacity of frozen or vitrified stallion sperm. Firstly, it was demonstrated that warming vitrified straws in a water bath at 60ºC / 5s can be used as an alternative to the conventional warming method, by immersion in a pre-warmed extender at 43ºC. Subsequently, the fertilizing capacity of the vitrified and frozen sperm was evaluated using heterologous IVF procedures, using cattle oocytes. Vitrification resulted in greater values ($P < 0.05$) than freezing for the number of bound sperm (1.36 ± 0.3 and 0.69 ± 0.2, respectively). There were no differences between frozen or vitrified sperm in pronuclear formation (26h. post-insemination-hpi; 14.08 ± 4.2% and 22.78 ± 4.8%, respectively) or cleavage rate (32.77 ± 4.3% and 39.66 ± 4.6%, respectively).

In conclusion, according to the results obtained in this Doctoral Thesis, vitrification of stallion sperm using non-permeable CPAs can be used as an alternative to conventional sperm freezing.

INTRODUCTION

INTRODUCTION

Conservation involves both *in vivo* maintenance of genetic diversity in form of live animals and *in vitro* storage of genetic material. Cryopreservation is a method for in vitro storage of biological material without deterioration for an indefinite period of time (Mazur, 1985). It is based on the collection and freezing of semen, oocytes, embryos or tissues for potential future use in breeding. The cryopreserved stallion sperm can be used for several procedures related to equine reproductive technologies, such as artificial insemination or intracytoplasmic sperm injection (ICSI) (Squires et al., 1996). Cryopreservation techniques have been evolving since spermatozoa were cryopreserved for the first time (Polge et al., 1949). This has been possible due to the increased understanding of the causes of cryo-injury. Slow cooling rates were used in the first sperm cryopreservation protocols. In slow freezing, cells are cooled below freezing point, where spontaneous ice nucleation will take place between -5ºC and -15ºC. After that, ice crystals will grow rapidly, leaving some channels called as the unfrozen fraction, where the cells and solutes are enclosed. It is well known that this conventional freezing method leads to structural and functional damage in the equine sperm, as a consequence of ice crystals formation and osmotic imbalance (Peña et al., 2011).

In order to protect sperm cells from cryo-injury during slow freezing, cryoprotectants agents (CPAs) have been used. In this sense, cryopreservation of stallion sperm has been traditionally performed by slow freezing methods, using permeable CPAs (Wu et al., 2015). When cells are maintained in a medium which contains permeable CPAs, water will leave the cells because of the osmotic pressure differences, while at the same time, CPAs will penetrate into the cells. Therefore, cells will regain its original volume, replacing their intracellular water with CPAs. Thus, the amount of ice crystals formation is lower in comparison to freezing without CPAs. However, some permeable CPAs are toxic for the sperm cell, reducing the success of cryopreservation (Fahy et al., 2010). Permeable CPAs exert their toxic effect as soon as the equilibration process starts. Particularly, glycerol, one of the most commonly used CPA, causes a great osmotic imbalance (Morris et al., 2007), due to his relatively high molecular weight (MW= 92.09 g/mol). Others CPAs, with lower molecular weight and greater coefficient of membrane permeability, such as dimethyl sulfoxide (MW= 78.13 g/mol), ethylene glycol (MW= 62.07 g/mol) or dimethylformamide (MW= 73.1 g/mol), have been introduced as an alternative to glycerol (Squires 2004; Alvarenga 2005). They penetrate faster the plasma membrane, causing a lower osmotic damage in comparison to glycerol (Squires et al., 2004; Medeiros et al., 2002). Nevertheless, these CPAs have other toxic effects at high concentration, causing denaturation and aggregation of proteins (Fahy et al., 2010; Arakawa et al., 1990; Giugliarelli et al., 2016).

Searching for alternative extenders to improve equine sperm cryopreservation, permeable CPAs have been combined with other agents, such as carbohydrates (Koshimoto et al., 2002; Aisen et al., 2002), proteins (Uysal et al., 2007), antioxidants (Contreras et al., 2020), lipids (Hartwig et al., 2014), aminoacids (Dorado et al., 2014), etc.

Sugars are the most commonly used carbohydrates for sperm cryopreservation (Ahmed et al., 2015; Woelders et al., 1997; Bucak et al., 2013). These sugars reduce the formation of large ice crystals in the extracellular medium, by increasing the viscosity of the extender (Sieme et al., 2015). They also preserve the membrane integrity through hydrogen bonding with the head polar groups of the lipids (Aisen et al., 2002; Panyaboriban et al., 2015; Uchida et al., 2007). Moreover, according to Isachenko et al. (2017), carbohydrate flux compensates for the change of osmotic pressure caused by the permeable CPAs during freezing and thawing: increasing this pressure during saturation by cryoprotectants (dehydration) and decreasing during removal of cryoprotectants (rehydration). On the other hand, different proteins,such as bovine serum albumin (BSA), have been usually added to the extender for animal sperm cryopreservation (Diaz-Jimenez et al., 2018a; Sanchez et al., 2011). BSA has an antioxidant function by removing reactive oxygen species (ROS) generated by oxidative stress (Uysal et al, 2005) and protecting the sperm membrane due to prevention of lipid peroxidation and stabilization of membrane proteins (Naijian et al., 2013; Cabrita et al, 2001).

Recently, a combination of sucrose (MW= 342.3 g/mol) with human or bovine serum albumin (BSA, MW= 66.5 kDa), has been applied for cryopreservation of human (Isachenko et al., 2008), dog (Sanchez et al., 2011), donkey (Diaz-Jimenez et al., 2018a), wild ibex (Pradiee et al., 2015) and rainbow trout (Merino et al., 2012) sperm, to reduce the osmotic imbalance and avoid the intrinsic toxicity and damage produced by permeable CPAs. The concentration of sucrose used in the extender is a key factor and there is species specificity regarding the most desirable concentration to use (Isachenko et al., 2008; Sanchez et al., 2011; Pradiee et al., 2015; Diaz-Jimenez et al., 2018a). Unfortunately, there is little information about the proper concentration of sucrose for stallion sperm freezing (Oldenhof et al., 2013) and no studies have been conducted combining sucrose and BSA as non-permeable agents. Therefore, the evaluation of different concentrations of these non-permeable cryoprotectants, as an alternative to glycerol for conventional freezing, should be address.

Nevertheless, fertility of stallion sperm after freezing and thawing is remarkably lower than in other animal species (Pande et al., 2018; Kirk et al., 2005). This suggests that stallion spermatozoa are especially sensitive to cryoinjury (Vidament et al., 1997). In order to enhance the outcome of the quality of cryopreserved stallion sperm, the search for another cryopreservation protocol is justified. Vitrification has been developed as an alternative method of cryopreservation. It is based on rapid freezing, in which viable cells undergo glass-like solidification (Pradiee et al., 2015). This technology requires large concentrations of permeable CPAs (Isachenko et al., 2003), which increase the viscosity of the medium and prevent extra- and intra-cellular ice formation during cooling and warming. This method has obtained successful results in vitrification of oocytes and embryos (Rall and Fahy, 1985). Unfortunately, this technology failed when applied to vitrification of sperm due to the lack of tolerance of these cells to such concentration of CPAs (Macias-Garcia et al., 2012; Oldenhof et al., 2017a). Recently, sperm vitrification was achieved by direct plunging of small volumes of sperm into liquid nitrogen without any permeable cryoprotectant (Isachenko et al., 2008). This technique has been termed “kinetic vitrification” (Isachenko et al., 2004a).

The ultra-rapid cooling rates achieved in this technique, prevents intracellular ice crystals formation. However, both vitrified and ice-forming regions may exist within the same extracellular solution. Thus, the "kinetic vitrification" for sperm processing, therefore, means something different as compared with conventional term for vitrification associated with oocytes and embryos (Katkov et al., 2006), where both the intracellular milieu and the extracellular environment must become vitrified, and there are inconsistent thoughts regarding the use of this terminology (Pradiee et al., 2015).

There are important factors for vitrification success, such as the semen concentration, volume or vitrification media (Arav et al., 2002), as well as the packaging and warming method (Diaz-Jimenez et al., 2019a; Mansilla et al., 2016). In previous studies of different species, vitrification has been conducted with success using the spheres method (Isachenko et al., 2008; Sanchez et al., 2011; Pradiee et al., 2015). However, this is a technique where a small volume of semen is utilized, and as a result, compromises the procedures used for artificial insemination of mares (Govaere et al., 2014). Moreover, it is a non-aseptic procedure, where cross-contamination can take place (Pomeroy et al., 2010). In this sense, it has been demonstrated than some non-enveloped virus and bacteria can survive at -196ºC (Tomlinson et al., 2008). For example, *Mycoplasma equigenitalium* is a cryo-stable pathogen, which is able to survive at -196ºC for 30 days (Bermudez et al., 1988). Therefore, a device that allows to cryopreserve sperm as well as prevents direct contact with LN_2 should be used.

The straw method has been used as an alternative to vitrification in spheres. This procedure provides for the opportunity to use a larger volume and concentration of sperm as well as for isolating sperm from LN_2, avoiding the risk of cross-contamination (Isachenko et al., 2017). Recently, sperm vitrification of donkey and human has been performed in 0.25 mL straws (Sanchez et al., 2012; Diaz-Jimenez et al. 2018b). This method has been proposed as an alternative to conventional freezing and vitrification in spheres. Moreover, sperm vitrification using 0.5 mL straws has been accomplished in human sperm (Isachenko et al., 2011); unfortunately, when this procedure was repeated in human, bovine and stallion sperm, the results were unsatisfactory (Katkov et al., 2012; Restrepo et al., 2019).

Vitrification of human sperm has been traditionally performed using samples with small concentrations of sperm, ranging from 1 to 15 x 10^6 sperm/mL (Sanchez et al., 2012; Mohamed et al., 2015). However, equine semen doses used for regular artificial insemination techniques need to contain spermatozoa in greater sperm concentration, usually from 50 to 500 x 10^6 sperm/mL (Heitland et al., 1996; Sieme et al., 2004). In this regard, previous studies of sperm vitrification in different species have included relatively greater concentrations of sperm (Pradiee et al., 2015; Diaz-Jimenez et al., 2019a). Unfortunately, there is no consensus about the optimal sperm concentration for sperm vitrification in animal species, varying from 2 to 1148 x 10^6 sperm/mL (Sanchez et al., 2011; Daramola et al., 2016). In this sense, vitrification success in different species can be related to the sperm concentration. Therefore, the proper sperm concentration for vitrification of stallion sperm should be studied.

The addition of carbohydrates to the extender results in a greater viscosity of the solution, which difficult ice crystal formation, therefore, enhancing the vitrification process (Arav et al., 2002). Sucrose has been the carbohydrate most commonly used for sperm vitrification in different species (Isachenko et al., 2008; Sanchez et al., 2011; Diaz-Jimenez et al., 2018b; Bóveda et al., 2018). Recently, other carbohydrates, such as trehalose (MW= 378.33 g/mol) and raffinose (MW= 594.52 g/mol), have been also evaluated for sperm vitrification in human (Schulz et al., 2017), mice (Horta et al., 2017) and dog species (Caturla-Sánchez et al., 2018). The optimal concentration of each sugar appears to be species-specific. In this regard, Iberian ibex sperm (Pradiee et al., 2015) appear to be more sensitive to relatively greater concentration of carbohydrates than dog (Caturla-Sánchez et al., 2018) or donkey sperm (Diaz-Jimenez et al., 2017; Diaz-Jimenez et al., 2019a).

On the other hand, proteins such as human serum albumin (Isachenko et al., 2004a) and BSA (Diaz-Jimenez et al., 2018b; Pradiee et al., 2015) are used for sperm vitrification to increase the viscosity of the medium and the glass transition temperature (Isachenko et al., 2004a). Serum of cow milk contains BSA as well as β-lactoglobulin, that have also demonstrated a protective effect on stallion sperm during cooling (Batellier et al., 1997). There is a compound for commercial extenders called Pronexcell, which consists on a mixture of serum milk proteins, containing β-lactoglobulin and BSA.The protective effects of these proteins make them attractive for stallion sperm vitrification. However, the proper concentration of Pronexcell for stallion sperm vitrification remains unknown. Other additives used for sperm preservation are the low-density lipoproteins (LDL), present in egg yolk plasma (Peruma, 2018). These lipoproteins can replace the phospholipids of the membranes, which are lost during cryopreservation, and preserve intactness of sperm membranes (Peruma, 2018). To the best of our knowledge, LDL have never been added to extenders for sperm vitrification. In this sense, the use of different non-permeable agents and their optimal concentrations should be address for each vitrification method (spheres and straws) for stallion sperm.

The warming regimen for the contents in 0.25 mL vitrified straws consists of immersion of the unsealed straws in a commercial milk-based extender at 43ºC (Diaz-Jimenez et al., 2017). After imposing this procedure, it is necessary to centrifuge the solution to concentrate the sperm for maintaining viable sperm after thawing. The sperm centrifugation procedure results in an additional cost and is time-consuming. To omit this process, the warming procedure could be performed by immersion of the straw in a water bath because this procedure was previously utilized in a study in which there was thawing of vitrified ram sperm with there being sperm viability after warming (Zilli et al., 2018). It is important to consider that the warming procedure is one of the most critical steps for vitrification success (Mazur and Seki, 2011). If low warming rates are used, ice crystals can form while the temperature is between the vitrification temperature and the freezing point of the solution (Seki and Mazur, 2008). In previous studies of sperm vitrification, higher warming velocities (higher temperature and lower time of exposure) obtained better sperm motility and plasma membrane integrity than lower warming rates (Mansilla et al., 2016; Pradiee et al., 2017).

These findings may be the reason for the positive results in previous studies when there was thawing of vitrified sperm of different wild ruminants that were stored in spheres when there was warming at temperatures as great as 60ºC to 65ºC (Pradiee et al., 2018; O'Brien et al., 2019). Moreover, vitrified 0.5 mL straws of donkey sperm have been warmed also in a water bath at 70ºC (Diaz-Jimenez et al., 2019b). To the best of our knowledge, these temperatures have not been evaluated for warming of vitrified stallion sperm, either using 0.25 mL or 0.5 mL straws.

On the other hand, sperm selection techniques have been developed to obtain the good quality sperm from the remaining sperm population (Morrell et al., 2009). Different sperm selection techniques have usually been performed before vitrification (Isachenko et al., 2008; Pradiee et al., 2015), but not after warming. In conventional sperm freezing, the use of colloid single layer centrifugation has reported an improvement in equine sperm quality after thawing (Ortiz et al., 2015); therefore, those methods could enhance the outcome of vitrification in large volumes, up to 0.5 mL, after warming.

The semen analysis and *in vitro* fertilization (IVF) represent the most adequate approaches for evaluation of the fertilizing capacity of a sperm sample *in vitro* because these methods include evaluation of gamete interactions, sperm penetration, pronuclear formation and early embryo development (Brahmkshtri et al., 1999). It is well known that homologous IVF evaluations are unsuccessful to evaluate the fertilizing ability of stallion sperm (Choi et al., 1994; Hinrichs et al., 2002; Roasa et al., 2007). The small IVF rates are related to the lack of sperm penetration into horse oocytes *in vitro* (Sessions-Bresnahan et al., 2014). These suboptimal IFV rates could be the result of either incomplete sperm capacitation *in vitro* (Tremoleda et al., 2003) or inadequate maturation of oocytes *in vitro* (Hinrichs et al., 2002). To assess the fertilizing capacity of stallion sperm, the use of the heterologous IVF procedure may be an effective method. This method has been successfully performed to assess the capacity of sperm to fertilize oocytes *in vitro* in different species, including red deer, wild goats, sheep and dolphins (Soler et al., 2008; Sánchez-Calabuig et al., 2015; Pradiee et al., 2018; Galarza et al., 2019). There was evaluation of conventionally frozen stallion sperm, utilizing the heterologous IVF procedures using either hamster (Matsukawa et al., 2002), pig (Balao da Silva et al., 2013) or bovineo ocytes (Sessions-Bresnahan et al., 2014; de Vasconcelos Franco et al., 2016; Al-Essawe et al., 2018). To the best of our knowledge, the fertilizing capacity of vitrified stallion sperm has not been previously assessed using heterologous oocytes for IVF procedures.

With this background, in order to develop the stallion sperm vitrification technique as an alternative to conventional techniques of cryopreservation, different experiences should be carried out assessing each important factor for vitrification success.

OBJETIVOS

OBJETIVOS

En la presente Tesis Doctoral se plantea como objetivo general el desarrollar la técnica de la vitrificación para la criopreservación de esperma de caballo como alternativa a las técnicas convencionales de congelación. Para ello, se plantearon distintos objetivos específicos desarrollados en cada una de las siete publicaciones incluidas en el compendio:

OBJETIVO 1

Evaluar el empleo de sacarosa como estrategia para evitar el uso de crioprotectores permeables en la congelación de esperma de caballo.

Este objetivo ha sido abordado en el **Capítulo 1**, y pretendió examinar el efecto de distintas concentraciones de sacarosa en comparación con un diluyente a base de glicerol, para la congelación convencional de esperma de caballo.

***C. Consuegra**, F. Crespo, M. Bottrel, I. Ortiz, J. Dorado, M. Diaz-Jimenez, B. Pereira, M. Hidalgo. Stallion sperm freezing with sucrose extenders: A strategy to avoid permeable cryoprotectants. Animal Reproduction Science (2018), 191, 85-91.*

OBJETIVO 2

Desarrollar el método de vitrificación de esperma de caballo en esferas.

Este objetivo ha sido abordado en el **Capítulo 2,** y se centró en la valoración del efecto de diferentes concentraciones de crioprotectores no permeables, así como la temperatura del periodo de equilibrado sobre la calidad del esperma tras la vitrificación, en comparación con la congelación convencional empleando glicerol.

*H. Hidalgo, **C. Consuegra**, J. Dorado, M. Diaz-Jimenez, I. Ortiz, B. Pereira, R. Sanchez, F. Crespo. Concentrations of non-permeable cryoprotectants and equilibration temperatures are key factors for stallion sperm vitrification success. Animal Reproduction Science (2018), 196, 91-98.*

OBJETIVO 3

Valorar la vitrificación de esperma de caballo en grandes volúmenes empleando pajuelas de 0,5 mL.

Este objetivo ha sido abordado en el **Capítulo 3,** y profundizó en la valoración de la vitrificación de grandes volúmenes de esperma, atendiendo al efecto de diferentes métodos de calentamiento y a la selección espermática mediante coloides, en comparación con la vitrificación de pequeños volúmenes y la congelación convencional.

***C. Consuegra**, F. Crespo, J. Dorado, M. Diaz-Jimenez,B. Pereira, I. Ortiz, R. Arenas, J.M. Morrell, M. Hidalgo.Vitrification of large volumes of stallion sperm in comparison to spheres and conventional freezing: effect of warming procedures and sperm selection. Journal of Equine Veterinary Science (2019), 83, 102680.*

OBJETIVO 4

Desarrollar un protocolo de vitrificación de esperma de caballo en pajuelas de 0,25 mL.

Este objetivo ha sido abordado en el **Capítulo 4**, e incluye los siguientes capítulos:

Capítulo 4.1. Comparar diferentes diluyentes a base de sacarosa para la vitrificación de esperma en pajuelas de 0,25 mL.

***C. Consuegra**, F. Crespo, J. Dorado, I. Ortiz, M.Diaz-Jimenez, B. Pereira, M. Hidalgo. Comparison of different sucrose-based extenders for stallion sperm vitrification in straws. Reproduction in Domestic Animals (2018), 53, 59-61.*

Capítulo 4.2. Determinar la concentración y volumen de esperma, así como el uso de distintos azúcares para la vitrificación de esperma de caballo en pajuelas de 0,25 mL, en comparación con la congelación convencional.

C. Consuegra, *F. Crespo, J. Dorado, M. Diaz-Jimenez, B. Pereira, I. Ortiz, M. Hidalgo. Vitrification of stallion sperm using 0.25 mL straws: Effect of volume, concentration and carbohydrates (sucrose/trehalose/raffinose). Animal Reproduction Science (2019), 206, 69-77.*

Capítulo 4.3. Valorar el efecto de la adición de lipoproteínas de baja densidad y proteínas del suero de la leche para la vitrificación de esperma de caballo en pajuelas de 0,25 mL.

C. Consuegra, *F. Crespo, J. Dorado, M. Diaz-Jimenez, B. Pereira, M. Hidalgo. Low-density lipoproteins and milk serum proteins improve the quality of stallion sperm after vitrification in straws. Reproduction in Domestic Animals (2019), 54, 86-89.*

OBJETIVO 5

Evaluar la capacidad fecundante del esperma vitrificado mediante fecundación *in vitro* (FIV) heteróloga.

Este objetivo ha sido abordado en el **Capítulo 5**, y consistió en determinar el método óptimo de calentamiento de muestras vitrificadas de esperma de caballo, así como valorar la capacidad fecundante de las muestras de esperma vitrificadas y congeladas mediante fecundación *in vitro* (FIV) heteróloga.

***C. Consuegra**, F. Crespo, J. Dorado, D. Rizos, M.J. Sánchez-Calabuig, P. Beltrán-Breña, S. Pérez-Cerezales, M. Diaz-Jimenez, B. Pereira, M. Hidalgo. Fertilizing capacity of vitrified stallion sperm assessed utilizing heterologous IVF after different semen warming procedures. Animal Reproduction Science (2020), 223, 106627.*

OBJECTIVES

OBJECTIVES

The general objective of this Doctoral Thesis is to develop the vitrification technique for the cryopreservation of stallion sperm, as an alternative to conventional cryopreservation techniques. For this purpose, different specific objectives were proposed which has been developed in each of the seven publications included in this Thesis:

OBJECTIVE 1

To determine the use of sucrose as a strategy to avoid permeable cryoprotectants for stallion sperm cryopreservation.

This objective has been addressed in **Chapter 1**, and which aimed to examine the effect of different concentrations of sucrose in comparison to a extender based on glycerol for conventional freezing of stallion sperm.

C. Consuegra, F. Crespo, M. Bottrel, I. Ortiz, J. Dorado, M. Diaz-Jimenez, B. Pereira, M. Hidalgo. Stallion sperm freezing with sucrose extenders: A strategy to avoid permeable cryoprotectants. Animal Reproduction Science (2018), 191, 85-91.

OBJECTIVE 2

To develop the spheres method for stallion sperm vitrification.

This objective has been addressed in **Chapter 2**, which has been focused on the evaluation of the effect of different concentrations of non-permeable cryoprotectants and the temperature of the equilibration period on the quality of the sperm after vitrification in comparison with conventional freezing using glycerol.

*H. Hidalgo, **C. Consuegra**, J. Dorado, M. Diaz-Jimenez, I. Ortiz, B. Pereira, R. Sanchez, F. Crespo. Concentrations of non-permeable cryoprotectants and equilibration temperatures are key factors for stallion sperm vitrification success. Animal Reproduction Science (2018), 196, 91-98.*

OBJECTIVE 3

To develop the vitrification of large volume of stallion sperm using 0.5 mL straws.

This objective has been addressed in **Chapter 3**, which has been focused on the assessment of vitrification of large volumes, and the effect of different warming procedures and sperm selection using single-layer centrifugation in comparison to vitrification in spheres and conventional freezing

***C. Consuegra**, F. Crespo, J. Dorado, M. Diaz-Jimenez,B. Pereira, I. Ortiz, R. Arenas, J.M. Morrell, M. Hidalgo.Vitrification of large volumes of stallion sperm in comparison to spheres and conventional freezing: effect of warming procedures and sperm selection. Journal of Equine Veterinary Science (2019), 83, 102680.*

OBJECTIVE 4

To develop a protocol forstallion sperm vitrification in 0.25 mL straws.

This objective has been addressed in **Chapter 4**, which includes the following chapters:

Chapter 4.1. Comparison of different extenders based on sucrose for vitrification of sperm in 0.25 mL straws.

C. Consuegra, *F. Crespo, J. Dorado, I. Ortiz, M. Diaz-Jimenez, B. Pereira, M. Hidalgo Comparison of different sucrose-based extenders for stallion sperm vitrification in straws. Reproduction in DomesticAnimals (2018), 53, 59-61.*

Chapter 4.2. To determine the concentration and volume of sperm, as well as the use of sugars for the vitrification of stallion sperm in 0.25 mL straws, and to compare this vitrification method with conventional freezing.

C. Consuegra*, F. Crespo, J. Dorado, M. Diaz-Jimenez, B. Pereira, I. Ortiz, M. Hidalgo. Vitrification of stallion sperm using 0.25 mL straws: Effect of volume, concentration and carbohydrates (sucrose/trehalose/raffinose). Animal Reproduction Science (2019), 206, 69-77.*

Chapter 4.3. To assess the effect of the addition of low-density lipoproteins and milk serum proteins for stallion sperm vitrification in 0.25 mL straws.

C. Consuegra*, F. Crespo, J. Dorado, M. Diaz-Jimenez, B. Pereira, M. Hidalgo. Low-density lipoproteins and milk serum proteins improve the quality of stallion sperm after vitrification in straws. Reproduction in Domestic Animals (2019), 54, 86-89*

OBJECTIVE 5

To evaluate the fertilizing capacity of vitrified stallion sperm by heterologous *in vitro* fertilization (IVF).

This objective has been addressed in Chapter 5 and consisted of the determination of the optimal method of warming of vitrified semen samples; and assessing the fertilizing capacity of vitrified and frozen sperm samples by heterologous *in vitro* fertilization (IVF).

C. Consuegra*, F. Crespo, J. Dorado, D. Rizos, M.J. Sánchez-Calabuig, P. Beltrán-Breña, S. Pérez-Cerezales, M. Diaz-Jimenez, B. Pereira, M. Hidalgo. Fertilizing capacity of vitrified stallion sperm assessed utilizing heterologous IVF after different semen warming procedures. Animal Reproduction Science (2020), 223, 106627.*

CHAPTERS

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Stallion sperm freezing with sucrose extenders: A strategy to avoid permeable cryoprotectants

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ABSTRACT

The aim of this study was to assess different concentrations of sucrose-based extenders combined with bovine serum albumin (BSA) as an alternative to stallion sperm cryopreservation with permeable cryoprotectants. Semen samples (n = 16) were collected from six stallions. Sperm was cooled, filled in 0.5 mL straws and frozen in nitrogen vapor. Post-thaw sperm kinetic parameters, plasma and acrosome membrane integrity were statistically compared among treatments. In Experiment 1, extenders containing 1% of BSA and different concentrations of sucrose (mmol/L, M): 0, 50, 100, 250, 350 and 450 mM were compared. Use of sucrose [100 mM (S2)] resulted in greater values for most of the sperm kinetic parameters assessed ($P < 0.001$). There were no differences for plasma membrane integrity, except for when sucrose was used at 50 and 250 mM concentrations, and plasma membrane integrity was less ($P < 0.05$) when these concentrations were used than with the other sucrose concentrations. In Experiment 2, the selected sucrose extender (S2) was compared to an extender containing glycerol as permeable cryoprotectant. Use of the S2 extender resulted in a lesser proportion of sperm with denuded-acrosomes ($P < 0.05$) in comparison to use of glycerol and values for several kinetic parameters were also greater ($P < 0.05$) with use of S2. There were no significant differences for the other parameters assessed in this study. In conclusion, stallion sperm can be frozen in the absence of permeable cryoprotectants, using a combination of sucrose 100 mM with BSA-1% as alternative agents.

1. Introduction

It is well known that cryopreservation leads to structural and functional damage of stallion sperm, mainly due to osmotic stress: hyper- and hypotonic imbalance during freezing and thawing causes cell shrinkage and swelling (Pena et al., 2011). In addition, the toxicity of membrane permeable cryoprotective agents (CPAs) used to protect the sperm during freezing is one of the most important factors affecting cryopreservation success (Fahy, 2010). Permeable CPAs exert toxic effects as soon as the equilibration of solute process starts (Vidament et al., 2009) with glycerol causing a greater osmotic imbalance (Morris et al., 2007) due to its relatively greater molecular weight (MW = 92.09 g/mol). Furthermore, glycerol exerts a biochemical toxic action, causing depolymerisation of actin of the sperm cytoskeleton, compromising the capacity of sperm to adapt to osmotic stress (Macias Garcia et al., 2012).

Other CPAs, with lesser molecular weights and greater coefficients of membrane permeability, such as dimethyl sulfoxide

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(MW = 78.13 g/mol), ethylene glycol (MW = 62.07 g/mol) or dimethylformamide (MW = 73.1 g/mol), have been proposed as alternative CPAs instead of use of glycerol in extenders for stallion sperm (Squires et al., 2004; Alvarenga et al., 2005). Such alternative CPAs penetrate the plasma membrane faster resulting in less osmotic damage (Medeiros et al., 2002; Squires et al., 2004). Nevertheless, these CPAs have other toxic effects, causing denaturation and aggregation of proteins (Arakawa et al., 1990; Fahy, 2010; Giugliarelli et al., 2016).

Searching for alternative extenders to improve stallion sperm cryopreservation, permeable CPAs have been combined with non-permeable agents, such as sucrose (S) and trehalose (Aisen et al., 2002; Koshimoto and Mazur, 2002; De Oliveira et al., 2017). These sugars provide protection against cellular damage preventing extracellular ice crystals formation by increasing the viscosity of the extender and preserving the membrane integrity through hydrogen bonding with the phosphate head groups of lipid bilayer (Aisen et al., 2002; Uchida et al., 2007; Panyaboriban et al., 2015).

Recently, a combination of sucrose (MW = 342.3 g/mol) with human or bovine serum albumin (BSA, MW = 66.5 kDa), has been applied for vitrification of human (Isachenko et al., 2008), dog (Sanchez et al., 2011), donkey (Diaz-Jimenez et al., 2017), wild ibex (Pradiee et al., 2015) and rainbow trout (Merino et al., 2012) sperm, to reduce the osmotic imbalance and avoid the intrinsic toxicity and damage produced by permeable CPAs. The concentration of sucrose used in the extender is a key factor and there is species-specificity regarding the most desirable concentration to use (Isachenko et al., 2008; Sanchez et al., 2011; Pradiee et al., 2015; Diaz-Jimenez et al., 2017). Unfortunately, there is little information about the proper concentration of sucrose for stallion sperm freezing (Oldenhof et al., 2013) and, no studies have been conducted combining sucrose and BSA as non-permeable agents.

The aim of this study, therefore, was to evaluate different concentrations of sucrose-based extenders combined with BSA as an alternative to glycerol for stallion sperm cryopreservation.

2. Materials and methods

All procedures for these experiments were approved by the Ethical Committee on Animal Experimentation of the University of Cordoba, in compliance with the Regional Government of Andalusia (project no. 31/08/2017/105) and the Spanish law for animal welfare and experimentation (RD 53/2013).

All chemicals used were purchased from Sigma Aldrich (St. Louis, MO, USA) unless otherwise stated. The base medium used for sperm freezing was a commercial skimmed milk–egg-yolk based extender with or without glycerol (Gent, Minitube GmbH, Tiefenbach, Germany) with addition of different concentrations of BSA and sucrose (see experimental design). The base medium for sperm evaluation was INRA96 (IMV-Technologies, L'Aigle France).

2.1. Animals

Six healthy stallions of different breeds (four Pure Spanish Horses, one Hispano-Arab and one Anglo-Arabian), from 5 to 21 years old and with known fertility were used as semen donors. Animals were housed in individual paddocks placed at the Equine Breeding Center of the Spanish Army located in Avila, Spain (40.66°N, 4.70°W).

2.2. Semen collection and processing

Semen samples were collected using a Missouri-model artificial vagina with an in-line gel filter (Minitube GmbH, Tiefenbach, Germany) during the breeding season. From each animal, 2 or 3 ejaculates were collected on a regular basis of two collections per week, obtaining a total number of 16 ejaculates. Immediately after collection, the gel-free volume (mL) was measured in a graduated collecting tube. Sperm concentration (millions sperm/mL) was assessed with a sperm photometer (Spermacue; Minitube GmbH, Tiefenbach, Germany) and total and progressive sperm motility was objectively evaluated as subsequently described.

2.3. Sperm freezing and thawing

Sperm aliquots were frozen following a standard protocol with modifications (Hidalgo et al., 2017). Fresh semen was diluted in a ratio 1:1 (v:v) with INRA96 and then centrifuged 10 min at 600*g*. The sperm pellet was re-extended to a final concentration of 50×10^6 sperm/mL in each freezing extender (see experimental design). Osmolalities of all extenders (Table 1) were measured using a freezing-point digital micro osmometer Type 6 (Löser Messtechnik, Berlin, Germany). Afterwards, sperm was slowly cooled to 5 °C within 2 h. Each dilution was loaded into 0.5 mL plastic straws and frozen horizontally in racks with samples being positioned 4 cm above the surface of liquid nitrogen for 10 min, and then plunged into LN_2. After no less than 1 week of storage in LN_2 tanks, straws were thawed in a water bath at 37 °C for 30 s.

2.4. Post-thaw sperm analysis

2.4.1. Computer-assisted sperm motility analysis

Sperm motility was evaluated objectively by computer-assisted sperm analysis (SCA v.5.4.0.0, Microptic S.L., Barcelona, Spain). The settings of the system and methodology used were previously described (Hidalgo et al., 2017). Two 5 µL drops, with three randomly chosen microscopic fields were analyzed of each semen sample using a Makler counting chamber (Sefi Medical Instruments Ltd., Haifa, Israel). The following parameters were analyzed: total (TM, %) and progressive sperm motility (PM, %), curvilinear

Table 1
Osmolality (mOsm kg^{-1}) and pHvalues of the different extenders used in these experiments.

Freezing extender	Osmolality	pH
S0	353	6.90
S1	215	6.91
S2	281	6.94
S3	438	6.93
S4	583	6.87
S5	642	6.80
GLY	1001	6.85

S0 = base extender without sucrose; S1 = sucrose 50 mM (mmoles/L); S2 = sucrose 100 mM (mmoles/L); S3 = sucrose 250 mM (mmoles/L); S4 = sucrose 350 mM (mmoles/L); S5 = sucrose 450 mM (mmoles/L); All the sucrose-based extenders contained BSA at 1%; GLY: base extender with glycerol.

velocity (VCL, μm/s), straight-line velocity (VSL, μm/s), average path velocity (VAP, μm/s), linearity (LIN, as VSL/VCL, %), straightness (STR, as VSL/VAP, %), wobble (WOB, as VAP/VCL, %), amplitude of lateral head displacement (ALH, μm) and beat cross frequency (BCF, Hz).

2.4.2. Plasma membrane integrity

The integrity of plasma membrane was assessed using Vital Test® (Halotech DNA S.L., Madrid, Spain), following the manufacturer instructions. Briefly, a 10 μL aliquot of diluted sperm (25×10^6 sperm/mL) were placed on a microscope slide, and mixed with 1 μL of acridine orange and 1 μL of propidium iodide (PI). At least, 200 sperm were evaluated, considering green spermatozoa as intact membrane sperm. The percentage of sperm with intact membrane was recorded (IMS, %).

2.4.3. Acrosome membrane integrity

To evaluate acrosome integrity, the PI/peanut agglutinin-fluorescein isothiocyanate (FITC-PNA) double stain was performed on ethanol-fixed sperm smear as previously described (Dorado et al., 2014). At least 200 sperm on each slide were evaluated using an epifluorescence microscope (Olympus BX-40, Olympus U-RLF-T, Tokyo, Japan) with a 530 nm ex-citation filter at 1000 X magnification and classified as follows: 1) acrosome-intact sperm (AIS, %: acrosome region displayed green fluorescence; PI + /FICT-PNA +); 2) acrosome-reacted sperm (ARS, %: green fluorescent at the equatorial segment or no anterior acrosomal staining; PI + FICT-PNA ±) and 3) denuded-acrosome sperm (ADS, %: sperm that display only red fluorescence; PI + /FICT-PNA-).

2.5. Experimental design

2.5.1. Experiment 1: evaluation of different concentrations of sucrose combined with BSA

In this experiment, sperm were frozen using the commercial glycerol-free base extender for stallion sperm (Gent) adding 1% of BSA and water-diluted sucrose as previously described by Isachenko et al. (2012), to reach a final concentration of (mmol/L, mM): 0 (S0, control); 50 (S1); 100 (S2); 250 (S3); 350 (S4); and 450 (S5) mM. Post-thaw sperm parameters were assessed and compared among different sucrose concentrations.

2.5.2. Experiment 2: comparison between sucrose-BSA or glycerol extenders

Based on results in Experiment 1, the extender with the concentration of sucrose for which there were the most desirable results was selected and compared with the same base extender but using the commercial extender containing glycerol (GLY). After freezing and thawing, sperm were centrifuged and re-suspended in the base medium for sperm analysis. Post-thaw sperm parameters were compared between both treatments.

2.6. Statistical analysis

Analysis of the data was conducted using the Statistical Analysis Systems software v9.0 (SAS Institute Inc., Cary, NC, USA). A general linear model (PROC MIXED) with animals, ejaculates and treatments as fixed effects was performed. Duncan *post hoc* test was used to assess differences among treatments. Results were expressed as mean ± standard error of the mean (SEM). The level of significance was set at $P < 0.05$.

3. Results

Before freezing, all the ejaculates had the following physiological values for the routine stallion spermiogram: gel-free volume = 53.81 ± 7.19 mL, sperm concentration = 252.09 ± 21.62 millions sperm/mL, total sperm motility = 81.91 ± 2.05% and progressive sperm motility = 51.68 ± 2.71%.

Table 2
Sperm quality parameters of frozen-thawed semen samples (n = 16; six stallions) subjected to different concentrations of sucrose-based extenders.

Post-thaw sperm parameters	Freezing extenders					
	S0	S1	S2	S3	S4	S5
TM (%)	37.8 ± 2.7^{b}	29.3 ± 3.2^{c}	50.2 ± 2.5^{a}	46.4 ± 2.2^{a}	21.3 ± 1.7^{d}	10.8 ± 1.0^{e}
PM (%)	20.6 ± 1.7^{b}	13.6 ± 2.5^{c}	32.8 ± 2.5^{a}	24.8 ± 2.0^{b}	7.1 ± 1.1^{d}	2.1 ± 0.4^{e}
IMS (%)	72.2 ± 1.5^{a}	65.8 ± 1.6^{b}	71.9 ± 1.7^{a}	68.5 ± 1.1^{b}	72.7 ± 1.6^{a}	72.2 ± 1.5^{a}
VCL (μm/s)	64.3 ± 3.7^{a}	48.9 ± 2.6^{b}	69.8 ± 3.6^{a}	67.2 ± 4.8^{a}	45.5 ± 3.9^{b}	30.1 ± 2.1^{c}
VSL (μm/s)	47.5 ± 3.4^{ab}	35.3 ± 2.4^{c}	53.2 ± 2.8^{a}	44.9 ± 3.7^{b}	25.0 ± 3.4^{d}	13.3 ± 1.9^{e}
VAP (μm/s)	55.3 ± 3.7^{a}	40.7 ± 2.6^{b}	61.2 ± 3.4^{a}	53.0 ± 4.0^{a}	31.3 ± 3.7^{c}	17.9 ± 2.0^{d}
LIN (%)	61.2 ± 2.3^{a}	61.3 ± 2.1^{a}	66.9 ± 1.5^{a}	54.8 ± 2.3^{b}	42.0 ± 3.4^{c}	32.9 ± 3.0^{d}
STR (%)	73.6 ± 1.7^{ab}	74.3 ± 1.4^{ab}	77.5 ± 1.2^{a}	71.7 ± 1.7^{b}	63.6 ± 2.9^{c}	57.8 ± 2.9^{d}
WOB (%)	76.0 ± 1.7^{ab}	75.4 ± 1.6^{b}	80.3 ± 0.9^{a}	70.4 ± 1.8^{c}	59.3 ± 2.7^{d}	51.9 ± 2.4^{e}
ALH (μm)	1.7 ± 0.0^{bcd}	1.6 ± 0.1^{cd}	1.8 ± 0.1^{bc}	2.1 ± 0.1^{a}	1.8 ± 0.1^{b}	1.5 ± 0.1^{d}
BCF (Hz)	6.3 ± 0.3^{a}	5.7 ± 0.2^{ab}	6.5 ± 0.2^{a}	6.4 ± 0.3^{a}	5.3 ± 0.5^{b}	3.9 ± 0.4^{c}

S0 = base extender without sucrose; S0 = base extender without sucrose; S1 = sucrose 50 mM (mmoles/L); S2 = sucrose 100 mM (mmoles/L); S3 = sucrose 250 mM (mmoles/L); S4 = sucrose 350 mM (mmoles/L); S5 = sucrose 450 mM (mmoles/L). All the extenders contained bovine serum albumin at 1%. TM: Total Motility; PM: Progressive Motility; IMS: Plasma membrane integrity; VCL: Curvilinear velocity: VSL: Straight line velocity; VAP: Average path velocity; LIN: Linearity; STR: Straightness; WOB: Wobble; ALH: Lateral head displacement; BCF: Beat cross frequency. Values are expressed as mean ± standard error. Different superscripts (a–e) between treatments indicate differences at $P < 0.001$ except for IMS ($P < 0.05$).

3.1. Experiment 1: evaluation of different concentrations of sucrose combined with BSA

Data for the results obtained in this experiment are included in Table 2. Sperm motility was similar among treatments, however, was always greater when S2 was used ($P < 0.001$) except for ALH, which was greater with use of S3 ($P < 0.001$). There were no differences for membrane integrity, except for when sucrose was used at 50 (S1) and 250 (S3) mM resulting in lesser ($P < 0.05$) values for membrane integrity. Results from this study indicated that S2 was the extender of choice for Experiment 2.

3.2. Experiment 2: comparison between sucrose-BSA or glycerol extenders

There were no significant differences when S2 and GLY were used as the extender for most of the sperm parameters assessed, except for ALH, VCL, VAP and BCF for which values were greater when S2 was used alone (Table 3; $P < 0.05$). Additionally, the greatest values ($P < 0.05$) for denuded-acrosome sperm occurred with use of the GLY extender.

Table 3
Comparison of stallion sperm parameters (n = 16; 6 stallions) between freezing procedures using sucrose (S) or glycerol (GLY) as cryoprotectants.

Post-thaw sperm parameters	Freezing extenders	
	S2	GLY
TM (%)	49.8 ± 4.4 ^{a}	47.4 ± 4.0 ^{a}
PM (%)	26.4 ± 5.2 ^{a}	25.5 ± 3.6^{a}
IMS,(%)	69.4 ± 1.4 ^{a}	63.8 ± 4.0 ^{a}
AIS (%)	55.0 ± 5.8 ^{a}	38.7 ± 5.8 ^{a}
ARS (%)	17.1 ± 4.7 ^{a}	20.2 ± 4.2 ^{a}
ADS (%)	27.9 ± 3.1^{b}	41.1 ± 6.2^{a}
VCL (μm/s)	60.2 ± 5.3^{a}	50.2 ± 4.2^{b}
VSL (μm/s)	38.8 ± 3.7^{a}	34.5 ± 3.6^{a}
VAP (μm/s)	47.0 ± 4.6^{a}	40.2 ± 3.8^{b}
LIN (%)	55.9 ± 1.9^{a}	58.2 ± 2.1^{a}
STR (%)	72.7 ± 1.6^{a}	73.8 ± 1.5^{a}
WOB (%)	65.0 ± 6.2^{a}	72.8 ± 1.7^{a}
ALH (μm)	2.1 ± 0.1^{a}	1.7 ± 0.1^{b}
BCF (Hz)	6.6 ± 0.3^{a}	6.0 ± 0.3^{b}

S2 = sucrose 100 mM (mmoles/L) + 1% bovine serum albumin; GLY: base extender with Glycerol. TM: Total Motility; PM: Progressive Motility; IMS: Plasma membrane integrity; AIS: Acrosome-intact sperm; ARS: Acrosome-reacted sperm; ADS: Acrosome-denuded sperm; VCL: Curvilinear velocity: VSL: Straight line velocity; VAP: Average path velocity; LIN: Linearity; STR: Straightness; WOB: Wobble; ALH: Lateral head displacement; BCF: Beat cross frequency. Values are expressed as mean ± standard error. Different superscripts (a–b) between treatments indicate differences ($P < 0.05$).

4. Discussion

In the present study, stallion sperm were successfully frozen using sucrose-BSA based extenders as an alternative to permeable CPAs, such as glycerol. Different techniques including post-thaw sperm dilution or simple centrifugation have been previously performed to minimize the presence of permeable CPAs so as to avoid toxic effects (Macias Garcia et al., 2009; Vidament et al., 2009; Rota et al., 2012; Ortiz et al., 2015). Nevertheless, use of these techniques did not lead to the expected results, probably due to glycerol having a harmful effect early in the solute equilibration process (Vidament et al., 2009). Hence, the use of sucrose and BSA as alternative CPAs was assessed in the present study, considering there are no previous studies of stallion sperm freezing with this combination of non-permeable agents.

Sucrose decreases extra and intracellular ice formation during cryopreservation (Sieme et al., 2015), and also protects the sperm membranes (Uchida et al., 2007). In stallions, there has been no evidence in recent studies of intracellular ice formation (Morris et al., 2007), therefore the damage caused by cryopreservation is probably due to osmotic stress (Pena et al., 2011). According to Isachenko et al. (2017) carbohydrate flux compensates for the change of osmotic pressure caused by the permeable cryoprotectants during freezing and thawing: increasing this pressure during saturation by cryoprotectants (dehydration) and decreasing during removal of cryoprotectants (rehydration). Sucrose, therefore, has been shown to have an important effect as an osmoprotectant during cryopreservation (Rosato and Iaffaldano, 2013; Pradiee et al., 2015). The BSA, however, has an antioxidant function by removing free radicals generated by oxidative stress, protecting the sperm membrane from lipid peroxidation during freezing and thawing (Uysal et al., 2005; Naijian et al., 2013). The BSA also contains free fatty acid that could be used as an energy source for sperm metabolism (Hossain et al., 2007). These actions improve the survival and fertility of cryopreserved sperm (Matsuoka et al., 2006; Nang et al., 2012).

The proper concentration of sucrose for cryopreservation varies among species, probably due to physiological and structural differences in the sperm cells (Woods et al., 2004; Varner, 2015; Varner et al., 2015). In Experiment 1 of the present study, different concentrations of sucrose were combined with a fixed proportion of BSA (1%), to determine if there were improvements in sperm cryopreservation (Sanchez et al., 2011; Merino et al., 2012; Naijian et al., 2013; Pradiee et al., 2015). Use of the extender containing 100 mM of sucrose (S2) resulted in the greatest values for most of the sperm kinetic parameters assessed, which is not consistent with findings previously reported in humans (Isachenko et al., 2008) and other animal species (Sanchez et al., 2011; Diaz-Jimenez et al., 2017). In these previous studies, greater concentrations of sucrose were needed for optimizing sperm kinetics. In the present study, greater concentrations of sucrose decreased sperm motility, probably due to the greater viscosity of the medium that probably impeded the normal sperm motility. Plasma membrane integrity was similar with use of the different sucrose extenders probably because osmolalities (215–642 mOsm/kg) of these extenders were within the range (160–871 mOsm/kg) where sperm could function as a linear osmometer adjusting cytoplasm volume and, therefore, avoiding plasma membrane damage (Ball and Vo, 2001; Pommer et al., 2002; Glazar et al., 2009). Interestingly, osmolality was greater in the control extender in comparison to extenders containing lesser concentrations of sucrose (S1 and S2), probably because water-diluted sucrose was used (Isachenko et al., 2012). According to these findings, use of the S2 extender allowed for achieving an effective protection of stallion sperm during freezing and thawing (Oldenhof et al., 2013).

In Experiment 2, S2 extender was compared to a commercial glycerol-based extender for sperm freezing (GLY), with the same composition but containing glycerol instead of sucrose and BSA. Interestingly, post-thaw sperm parameters obtained with use of S2 were similar or even superior to when GLY was added, in particular for the integrity of acrosomal membrane. Glycerol depolymerizes F-actin of cytoskeleton (Macias Garcia et al., 2012), which is a necessary cellular function to allow the fusion of plasma membrane and outer acrosomal membrane, promoting the acrosomal reaction (Watson, 2000). This consideration could explain the greater amount of denuded-acrosome sperm that were present with use of the GLY extender. Additionally, use of S2 s resulted in greater values for VCL, VSL, ALH and BCF than GLY. The VCL and VSL have been shown to be correlated with fertility; in particular, VCL appears to be important for the penetration of the zona pellucida of the oocyte (Olds-Clarke, 1996; Verstegen et al., 2002). The values for ALH and BCF indicate the vigor of flagellar beating which is important for sperm to progress through the cervical mucus and, therefore, for *in vitro* and *in vivo* fertilization to occur (Aitken et al., 1985; Verstegen et al., 2002).

The osmolality of GLY extender (1001 mOsm/kg) is far greater than the alternatives used in the present study, which are closer to the isosmolal concentration of the sperm (Ball and Vo, 2001). Osmolality's of extenders appear to be a major problem for sperm cryopreservation in stallions (Darr et al., 2016) because relatively greater osmolalities result in an impairment of sperm membranes during freezing and thawing (Ball and Vo, 2001; Nang et al., 2012). In previous studies, glycerol could not be completely removed from commercial extenders but there were reductions in its concentration in combination with use other cryoprotectants (De Oliveira et al., 2017). It is important to emphasize that glycerol could be harmful even at low concentrations for cryopreservation of stallion semen (Macias Garcia et al., 2012; Oldenhof et al., 2017). In Experiment 2 of the present study, samples were centrifuged after thawing to remove most of the cryoprotectants thereby avoiding at least some of the potential detrimental effect on sperm cells. Considering the results of the present study, however, the harmful effect of glycerol was not decreased by use of centrifugation. This might have occurred because of the apparently irreversible intracellular damage exerted during the equilibration period before freezing (Vidament et al., 2009; Ortiz et al., 2015; Diaz-Jimenez et al., 2017).

In conclusion, stallion sperm can be frozen in the absence of permeable cryoprotectants, using a combination of sucrose 100 mM and 1% of BSA as alternative agents.

Conflict of interest

The authors declare no conflicts of interest.

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Concentrations of non-permeable cryoprotectants and equilibration temperatures are key factors for stallion sperm vitrification success

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ABSTRACT

Vitrification is based on rapid freezing by direct exposure of sperm to liquid nitrogen (LN_2). This study evaluated the effect of non-permeable CPAs and equilibration temperature on stallion sperm quality after vitrification. In Experiment 1, different concentrations of sucrose (20, 50, 100 mM; mmol/L) and bovine serum albumin (BSA 1%, 5%, 10%) were compared including different temperatures for the equilibration (≈22 °C or 5 °C). Vitrification was performed dropping 30 µl sperm suspension directly into LN_2. In Experiment 2, conventional sperm freezing using 2.2% of glycerol in 0.5 ml straws, frozen in LN_2 vapours, was compared to the sucrose and BSA extenders (and its combination) producing the most desirable results. Sperm motility, plasma membrane and acrosome integrity were statistically compared between treatments. Vitrification after sperm cooling at 5 °C with sucrose 20 mM (S20) or BSA 1% (BSA1) resulted in the greatest values (mean ± SEM) for most of the sperm variables assessed. With use of the combination (S20 + BSA1/5 °C), there were greater values ($P < 0.001$) than freezing with glycerol for total (55.67 ± 2.99 vs 35.41 ± 2.96) and progressive sperm motility (38.32 ± 3.05 vs 14.42 ± 1.80), plasma membrane integrity (66.61 ± 2.69 vs 49.16 ± 2.60), intact-acrosomes (49.19 ± 2.60 vs 14.91 ± 1.57) and most of the kinetics assessed, respectively. In conclusion, stallion sperm can be vitrified after cooling at 5 °C using a combination of 20 mM sucrose and 1% BSA based extender and this is a promising alternative compared with conventional sperm freezing using glycerol.

1. Introduction

Cryopreservation of stallion sperm has been traditionally performed by slow freezing methods, including the use of different permeable cryoprotectants (CPAs); (Wu et al., 2015). With this technique, sperm are usually cooled (equilibration period) before freezing in nitrogen vapours or using a programmable biofreezer at slow rates (Clulow et al., 2008).

Vitrification is an alternative method of cryopreservation, based on rapid freezing, in which viable cells undergo glass-like solidification (Pradiee et al., 2015). This technology requires large concentrations of permeable CPAs (Isachenko et al., 2003), which increase the viscosity of the medium and prevent intracellular ice formation during cooling and warming, obtaining successful results

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in vitrification of oocytes and embryos (Rall and Fahy, 1985). Unfortunately, this technology failed when applied to vitrification of sperm due to the lack of tolerance of these cells to such concentration of CPAs (Macias Garcia et al., 2012; Oldenhof et al., 2017a). Recently, sperm vitrification was achieved by direct plunging of small volumes of sperm into liquid nitrogen without any permeable cryoprotectant (Isachenko et al., 2008). This technique has been termed `kinetic vitrification´ because ultra-rapid cooling rates prevents the intracellular ice formation and promotes the glass-like solidification of the sperm (Isachenko et al., 2004b); however, both vitrified and ice-forming regions may exist within the same extracellular solution and could be difficult to differentiate between vitrification and ultra-rapid freezing (Shaw and Jones, 2003). The `kinetic vitrification´ for sperm processing, therefore, means something different as compared with conventional term for vitrification associated with oocytes and embryos (Katkov et al., 2006), where both the intracellular milieu and the extracellular environment must become vitrified and there are inconsistent thoughts regarding the use of this terminology (Pradiee et al., 2015).

Kinetic vitrification has been successfully used for human and sperm of different animal species (Isachenko et al., 2004b, 2008; Sanchez et al., 2011; Merino et al., 2012; Pradiee et al., 2015). Tested vitrification media included the combination of carbohydrates and proteins (Hossain and Osuamkpe, 2007; Schulz et al., 2017) instead of permeable CPAs, mainly glycerol, traditionally used for slow freezing. It has been termed as `cryoprotectant-free vitrification´ in some reports (Isachenko et al., 2004a). A combination of sucrose and bovine serum albumin (BSA), as non-permeable agents, has previously been used in conventional freezing of stallion and donkey sperm as a strategy to avoid the toxicity of permeating CPAs (Diaz-Jimenez et al., 2018; Consuegra et al., 2018). Vitrification is also a simpler and cost-effective technique which makes it attractive for cryopreservation of sperm in commercial laboratories or even in field conditions (Pradiee et al., 2015). In recent reports, however, there has been little information about application of this technique, particularly in non-human species.

The aim of the present study was to evaluate the effect of non-permeable CPAs and temperature during the equilibration period on stallion sperm quality after vitrification and warming in comparison to conventional sperm freezing with glycerol.

2. Materials and methods

This study was approved by the Ethical Committee for Animal Experimentation of the University of Cordoba, in compliance with the Regional Government of Andalusia (project no. 31/08/2017/105) and the Spanish law for animal welfare and experimentation (RD 53/2013). All chemicals used were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise is stated. The base medium used for sperm processing and freezing as control was INRA96 (IMV Technologies, L'Aigle, France) adding different concentrations of bovine serum albumin (BSA) and sucrose (S) for sperm vitrification (see Experiment 1) or glycerol (GLY) for conventional slow freezing (see Experiment 2).

2.1. Semen collection and processing

Semen was collected from six clinically healthy stallions of different breeds (aged 6–15 years) using an artificial vagina in the presence of a mare in oestrus. Semen was collected from each animal twice a week on different sampling occasions obtaining fifteen ejaculates for Experiment 1 and eighteen for Experiment 2 (n = 33; 2–4 ejaculates per animal in each experiment). All the semen samples had at least a gel-free volume > 20 mL, sperm concentration > 146 × 10^6 sperm/mL, total sperm motility > 76.6% and progressive sperm motility > 49.9% evaluated as previously described (Ortiz et al., 2014). Fresh semen was diluted in a ratio 1:1 (v:v) with INRA96 and then aliquots were centrifuged 10 min at 600 × g (Alvarenga et al., 2012). The sperm pellets were re-extended to a final concentration of 50 × 10^6 sperm/mL in the control base medium (C, INRA96) adding BSA or S for sperm vitrification or GLY for slow freezing (see experimental design). After that, sperm samples were slowly frozen or vitrified as subsequently described.

2.2. Vitrification and warming of sperm

Sperm vitrification was conducted in spheres (small volumes) as previously described by Isachenko et al. (2008). A styrofoam box loaded with liquid nitrogen (LN_2) was used. Briefly, 30 µl droplets of the sperm suspension with the vitrification media (S or BSA) were plunged directly into LN_2 at distance of 10 cm from the surface. After contact with the LN_2 a sphere immediately forms and floats on the surface. It is important to avoid the placement of the spheres into a drop of greater volume. When solidification occurs (after about 24 s), the spheres descend to the bottom and can be easily collected by dissecting forceps. Spheres were packaged into 1.8 ml cryotubes maintained in LN_2 through the entire process (Arraztoa et al., 2017). Warming was performed after at least 24 h of storage in LN_2. Spheres were quickly submerged one by one (not more than five spheres) into 2 ml of INRA96 pre-warmed to 42 °C and gentle vortexing for 5 s. Post warm sperm suspension was centrifuged at 600 × g/10 min and the sperm pellet was resuspended with INRA96 to a final concentration of 25 × 10^6 sperm/ml for sperm evaluation.

2.3. Freezing and thawing

Semen samples were frozen following a standard protocol for stallions with modifications (Hidalgo et al., 2017). Briefly, diluted sperm pellets with the freezing medium with 2.2% glycerol (GLY) were slowly cooled to 5 °C within 2 h (Hidalgo et al., 2014) and then loaded in 0.5 ml plastic straws. The straws were frozen horizontally in racks placed 4 cm above the surface of liquid nitrogen (LN_2) for 10 min and placed into LN_2 tanks. After at least 24 h of storage in LN2, straws were thawed by immersion in a 37 °C water bath for 30 s, centrifuged (600 × g/10 min) and re-suspended with INRA96 for semen analysis.

2.4. Post-thaw sperm evaluation

Sperm motility was objectively evaluated using the Sperm Class Analyzer (SCA v5.01, Microptic S.L., Barcelona, Spain). The features of this system have been described previously (Ortiz et al., 2014). The following kinematic variables were recorded: total (TM, %) and progressive motility (PM, %), curvilinear (VCL, μm/s), straight line (VSL, μm/s) and average path velocities (VAP, μm/s), linearity (LIN, VSL/VCLx100), straightness (STR, VSL/VAPx100), wobble (WOB, VAP/VCLx100), amplitude of lateral head displacement (ALH, μm) and beat cross frequency (BCF, Hz). The integrity of plasma membrane was assessed using Vital test (Halotech SL, Madrid, Spain) under fluorescence microscopy (Cortes-Gutierrez et al., 2008). The percentage of sperm with intact plasma membrane was recorded (IMS, %). Acrosome integrity was evaluated using the propidium iodide (PI)/peanut agglutinin–fluorescein isothiocyanate (FITC-PNA) double stain (Dorado et al., 2013). Ethanol-permeabilized stallion sperm were classified into three groups: acrosome-intact sperm (AIS, %) acrosome-reacted sperm (ARS, %)and acrosome-denuded sperm (ADS, %).

2.5. Experimental design

2.5.1. Experiment 1. Effect of different sucrose and BSA concentrations for stallion sperm vitrification at different equilibration temperatures

After semen processing, sperm were extended in the control base medium (C; 306 mOsm/Kg) adding sucrose (S) at the following concentrations (mmol/L): 20 mM (S20, 343 mOsm/kg), 50 mM (S50, 392 mOsm/kg) and 100 mM (S100, 406 mOsm/kg); or BSA at final concentration of 1% (BSA1, 310 mOsm/kg), 5% (BSA5, 332 mOsm/kg) and 10% (BSA10, 343 mOsm/kg). Sperm suspensions were immediately vitrified after 5 min of equilibration at room temperature (≈ 22 °C) or after 2 h of cold-storage (5 °C) (Hidalgo et al., 2014). Post-thaw sperm variables were assessed as described before and compared between S or BSA treatments at different temperatures.

2.5.2. Experiment 2. Comparison between conventional freezing and sperm vitrification

The use of sucrose (20 mM) and BSA concentration (1%) resulted in the most desirable results in Experiment 1 (5 °C) and the combination of both cryoprotectants (S20 + BSA1, 349 mOsm/kg) was compared to conventional slow freezing using the base medium adding 2.2% of glycerol (GLY, 1150 mOsm/kg). Subsequently, values for post-thaw sperm variables were compared between treatments.

2.6. Statistical analysis

Results were expressed as mean ± standard error of the mean (SEM). Differences between mean values were analysed using a general linear model (PROC GLM) followed by the Duncan test with animals and ejaculates as random factors. All analyses were performed with SAS statistic package (Institute Inc., Cary, NC, USA). The level of significance was set at $P < 0.05$.

3. Results

3.1. Experiment 1. Effect of different sucrose and BSA concentrations for stallion sperm vitrification at different equilibration temperatures

Vitrification performed at 5 °C with a sucrose concentration of 20 mM (S20) resulted in the greatest values for plasma membrane integrity (IMS) and most of the sperm motility variables assessed (TM, PM, VCL, VSL, and VAP) in comparison to the other sucrose

Table 1
Comparison of sperm variables after vitrification of stallion sperm (5 °C/22 °C) with sucrose-based extenders.

Variables	Vitrification at 5 °C				Vitrification at 22 °C			
	C	S20-mM	S50-mM	S100-mM	C	S20-mM	S50-mM	S100-mM
TM (%)	38.51 ± 2.88^{ab}	46.85 ± 4.10^{a}	45.13 ± 3.80^{ab}	42.70 ± 3.77^{ab}	37.36 ± 3.14^{ab}	36.03 ± 2.51^{ab}	34.58 ± 2.16^{b}	41.54 ± 3.51^{ab}
PM (%)	21.19 ± 2.70^{b}	32.92 ± 4.10^{a}	25.26 ± 2.78^{ab}	26.68 ± 3.21^{ab}	18.53 ± 3.16^{b}	20.51 ± 2.16^{b}	19.74 ± 2.06^{b}	22.82 ± 2.16^{b}
IMS (%)	55.46 ± 2.43^{ab}	60.83 ± 2.62^{a}	59.04 ± 2.13^{ab}	57.99 ± 3.54^{ab}	52.67 ± 2.83^{ab}	48.99 ± 2.22^{b}	50.42 ± 4.10^{ab}	48.86 ± 3.97^{b}
VCL (μm/s)	51.15 ± 5.01^{b}	67.55 ± 3.57^{a}	62.62 ± 4.09^{ab}	66.15 ± 4.27^{a}	50.57 ± 5.44 ^{b}	61.04 ± 4.51^{ab}	57.93 ± 4.19^{ab}	56.13 ± 3.01^{ab}
VSL (μm/s)	28.63 ± 3.57^{b}	40.07 ± 2.97^{a}	33.21 ± 3.23^{ab}	38.27 ± 2.95^{ab}	33.74 ± 3,66^{ab}	37.51 ± 3.19^{ab}	37.33 ± 3.60^{ab}	34.68 ± 2.87^{ab}
VAP (μm/s)	32.82 ± 3.91^{b}	45.88 ± 3.26^{a}	37.13 ± 4.21^{ab}	44.29 ± 3.31^{ab}	37.65 ± 3.75^{ab}	41.88 ± 3.41^{ab}	41.73 ± 3.91^{ab}	38.85 ± 2.60^{ab}
ALH (μm)	2.46 ± 0.17^{a}	2.71 ± 0.12^{a}	2.46 ± 0.22^{a}	2.75 ± 0.25^{a}	2.22 ± 0.26^{a}	2.62 ± 0.24^{a}	2.48 ± 0.17^{a}	2.83 ± 0.26^{a}
LIN (%)	54.47 ± 3.36^{a}	59.33 ± 3.01^{a}	53.05 ± 4,86^{a}	57.50 ± 3.33^{a}	59.34 ± 3.79^{a}	60.33 ± 3.79^{a}	63.69 ± 2.64^{a}	61.13 ± 2.68^{a}
STR (%)	86.12 ± 1.54^{a}	87.18 ± 1.25^{a}	80.63 ± 5.24^{a}	85.39 ± 2.36^{a}	87.18 ± 2.81^{a}	88.53 ± 1.91^{a}	89.14 ± 0.96^{a}	87.06 ± 4.03^{a}
WOB (%)	62.27 ± 3.32^{a}	67.41 ± 2.83^{a}	63.10 ± 3.95^{a}	66.73 ± 2.85^{a}	68.14 ± 2.46^{a}	67.71 ± 1.89^{a}	67.19 ± 4.98^{a}	68.66 ± 2.05^{a}
BCF (Hz)	9.87 ± 0.59^{a}	10.88 ± 0.52^{a}	10.02 ± 0.83^{a}	11.17 ± 0.47^{a}	10.24 ± 0.83^{a}	9.97 ± 0.59^{a}	10.27 ± 0.69^{a}	10.03 ± 0.79^{a}

C = control – base extender; S = sucrose at different concentrations (mmol/l). TM: Total Motility; PM: Progressive Motility; IMS: Plasma membrane integrity; VCL: Curvilinear velocity: VSL: Straight line velocity; VAP: Average path velocity; LIN: Linearity; STR: Straightness; WOB: Wobble; ALH: Lateral head displacement; BCF: Beat cross frequency; Values are expressed as mean ± standard error; Different superscripts between treatments indicate differences at $P < 0.05$ except for PM ($P < 0.001$).

Table 2
Comparison of sperm variables after vitrification of stallion sperm (5 °C/22 °C) with BSA-based extenders.

Variables	Vitrification at 5 °C				Vitrification at 22 °C			
	C	BSA1%	BSA5%	BSA10%	C	BSA1%	BSA5%	BSA10%
TM (%)	38.51 ± 2.88^{abc}	45.81 ± 2.65^{a}	44.01 ± 2.26^{ab}	35.92 ± 3.66^{abc}	37.36 ± 3.14^{abc}	31.53 ± 3.93^{bcd}	28.93 ± 3.67^{cd}	20.73 ± 3.45^{d}
PM (%)	21.19 ± 2.70^{bc}	30.16 ± 2.62^{a}	29.18 ± 2.99^{ab}	21.93 ± 2.87^{abc}	18.53 ± 3.16^{cd}	16.47 ± 3.04^{cd}	13.32 ± 2.77^{cd}	12.27 ± 2.19^{d}
IMS (%)	55.46 ± 2.43^{ab}	57.18 ± 2.74^{a}	55.33 ± 2.51^{ab}	46.98 ± 3.38^{bc}	52.67 ± 2.83^{ab}	42.52 ± 3.76^{cd}	39.18 ± 3.87^{cd}	36.62 ± 3.19^{d}
VCL (μm/s)	51.15 ± 5.01^{c}	76.78 ± 4.38^{ab}	82.97 ± 7.06^{a}	68.84 ± 7.62^{abc}	50.57 ± 5.44^{c}	62.95 ± 7.74^{abc}	52.78 ± 7.59 ^{c}	58.80 ± 8.14^{bc}
VSL (μm/s)	28.63 ± 3.57^{c}	44.54 ± 3.11^{ab}	52.62 ± 4.52^{a}	41.04 ± 4.99^{abc}	33.74 ± 3.66^{bc}	33.43 ± 4.55^{bc}	29.68 ± 4.98^{c}	34.37 ± 4.52^{bc}
VAP (μm/s)	32.82 ± 3.92^{c}	53.91 ± 3.86^{ab}	62.75 ± 5.59^{a}	51.44 ± 6.03^{ab}	37.65 ± 3.75^{bc}	43.65 ± 5.94^{bc}	37.73 ± 5.83^{bc}	43.88 ± 5.71^{bc}
ALH (μm)	2.46 ± 0.17^{ab}	2.85 ± 0.17^{a}	3.05 ± 0.35^{a}	2.67 ± 0.42^{ab}	2.22 ± 0.26^{ab}	2.27 ± 0.39^{ab}	1.75 ± 0.32^{b}	1.76 ± 0.32^{b}
LIN (%)	54.47 ± 3.36^{a}	58.35 ± 2.49^{a}	62.75 ± 2.73^{a}	53.56 ± 5.93^{a}	59.34 ± 3.79^{a}	49.23 ± 5.92^{a}	47.65 ± 6.72^{a}	54.67 ± 7.18^{a}
STR (%)	86.12 ± 1.54^{a}	83.10 ± 1.73^{ab}	84.15 ± 1.74^{a}	71.77 ± 6.86^{abc}	87.13 ± 2.81^{a}	74.65 ± 5.11^{abc}	66.95 ± 7.63^{bc}	63.69 ± 9.18^{c}
WOB (%)	62.27 ± 3.32^{a}	70.16 ± 2.71^{a}	74.39 ± 2.63^{a}	67.56 ± 5.97^{a}	68.14 ± 2.46^{a}	63.04 ± 5.57^{a}	61.6 ± 6.60^{a}	68.17 ± 6.89^{a}
BCF (Hz)	9.87 ± 0.59^{ab}	10.32 ± 0.43^{a}	8.56 ± 0.73^{abc}	7.58 ± 1.04^{bcd}	10.24 ± 0.83^{a}	7.05 ± 0.99^{cd}	5.55 ± 0.92^{d}	6.05 ± 1.07^{cd}

C = control − base extender; BSA = Bovine serum albumin at different concentrations (%). TM: Total Motility; PM: Progressive Motility; IMS: Plasma membrane integrity; VCL: Curvilinear velocity: VSL: Straight line velocity; VAP: Average path velocity; LIN: Linearity; STR: Straightness; WOB: Wobble; ALH: Lateral head displacement; BCF: Beat cross frequency; Values are expressed as mean ± standard error; Different superscripts between treatments indicate differences at $P < 0.001$ except for VAP, ALH and STR ($P < 0.05$).

Table 3
Comparison of stallion sperm variables between conventional freezing and selected vitrification procedures.

Sperm parameters	Conventional freezing	Vitrification procedures (5 °C)		
	GLY	S20-mM + BSA1%	S20-mM	BSA1%
TM (%)	35.41 ± 2.96^{c}	55.67 ± 2.99^{a}	45.62 ± 3.01^{b}	42.83 ± 2.66^{b}
PM (%)	14.42 ± 1.80^{c}	38.32 ± 3.05^{a}	25.99 ± 1.89^{b}	28.17 ± 2.71^{b}
IMS (%)	49.16 ± 2,60^{c}	66.61 ± 2.69^{a}	58.51 ± 2.84^{b}	57.28 ± 2.39^{b}
AIS (%)	14.91 ± 1.57^{c}	49.19 ± 2.60^{a}	45.33 ± 3.20^{a}	37.87 ± 2.92^{b}
ARS (%)	50.03 ± 1.36^{a}	30.43 ± 2.15^{c}	33.77 ± 1.62^{c}	38.71 ± 2.15^{b}
ADS (%)	35.06 ± 1.64^{a}	20.38 ± 2.17^{b}	20.90 ± 3.07^{b}	23.42 ± 2.59^{b}
VCL (μm/s)	43.87 ± 2.2^{b}	79.41 ± 5.69^{a}	71.53 ± 7.77^{a}	69.67 ± 3.59^{a}
VSL (μm/s)	24.92 ± 2.06^{c}	47.27 ± 3.74^{a}	39.19 ± 2.60^{b}	45.28 ± 2.97^{a}
VAP (μm/s)	28.87 ± 2.08^{c}	57.47 ± 4.46^{a}	46.50 ± 3.82^{b}	51.86 ± 3.31^{ab}
ALH (μm)	2.32 ± 0.09^{b}	2.96 ± 0.16^{a}	2.69 ± 0.14^{ab}	2.69 ± 0.15^{ab}
LIN (%)	55.59 ± 2.74^{a}	60.59 ± 3.25^{a}	60.19 ± 2.66^{a}	65.02 ± 2.06^{a}
STR (%)	84.95 ± 1.56^{a}	83.41 ± 3.03^{a}	85.43 ± 2.45^{a}	87.94 ± 1.32^{a}
WOB (%)	64.98 ± 2.29^{a}	72.22 ± 2.15^{a}	70.28 ± 1.94^{a}	73.84 ± 2.11^{a}
BCF (Hz)	10.04 ± 0.65^{a}	9.89 ± 0.31^{a}	10.67 ± 0.35^{a}	9.61 ± 0.44^{a}

C = control – base extender; S = sucrose at different concentrations (mmol/l); BSA = Bovine serum albumin at different concentrations (%); GLY = glycerol 2.2%. TM: Total Motility; PM: Progressive Motility; IMS: Plasma membrane integrity; AIS: Acrosome – intact sperm; ARS: Acrosome – reacted sperm; ADS: Acrosome – denuded sperm; VCL: Curvilinear velocity: VSL: Straight line velocity; VAP: Average path velocity; LIN: Linearity; STR: Straightness; WOB: Wobble; ALH: Lateral head displacement; BCF: Beat cross frequency; Values are mean ± standard error; Different superscripts between treatments indicate differences at $P < 0.001$ except for ALH ($P < 0.05$).

extenders. There were no significant differences when sucrose treatments were imposed for the remaining sperm variables evaluated (Table 1). In addition, sperm vitrification with BSA1 at 5 °C resulted in the greatest values for, TM, PM, IMS and most of the kinematic variables assessed (Table 2) in comparison to the other BSA extenders.

3.2. Experiment 2. Comparison between conventional freezing and sperm vitrification

The use of the combination of BSA1 and S20 (S20 + BSA1) for sperm vitrification at 5 °C resulted in the greatest values for TM (55.67 ± 2.99), PM (38.32 ± 3.05), IMS (66.61 ± 2.69), AIS (49.19 ± 2.60) and all the remaining kinetic sperm variables assessed in comparison to slow freezing with GLY and vitrification using BSA1 or S20. The use of slow freezing with GLY resulted in the lowest values for TM (35.41 ± 2.96), PM (14.42 ± 1.80) IMS (49.16 ± 2.60), AIS (14.91 ± 1.57) and the remaining kinetic variables assessed, except for STR, WOB, and BCF (Table 3).

4. Discussion

In the present study, vitrification of stallion sperm has been successfully performed using small volumes of sperm in a combination of sucrose and BSA based extenders. There were more desirable values for sperm variables after warming than with conventional freezing. Sperm vitrification has obtained not only better values for plasma membrane and acrosome integrity but also unexpected high values of 56% for total sperm motility and 38% for progressive sperm motility.

Previous studies showed that vitrification of small volumes (spheres) of human sperm in the absence of permeable CPAs resulted in similar sperm parameters after warming in comparison to conventional freezing (Isachenko et al., 2004a). In animal species, vitrification of sperm in spheres resulted in greater values for plasma membrane and acrosome integrity (Sanchez et al., 2011; Merino et al., 2012) but resulted in no motile sperm in boars (Arraztoa et al., 2017), 3% progressive motility in rabbits (Rosato and Iaffaldano, 2013) and 8% in rams (Arando et al., 2017). In a preliminary study in horses where vitrification was performed, there was limited preservation of sperm motility (progressive motility < 10%; (Pérez-Marín et al., 2017). Semen samples from dogs (Sanchez et al., 2011), fish (Merino et al., 2012), Iberian wild goats (Pradiee et al., 2015) and cats (Swanson et al., 2017) had greater sperm motility values after warming following the sphere method. In other animal species, the use of a combination of permeable and non-permeable CPAs resulted also in no motile sperm after vitrification in straws (Jiménez-Rabadán et al., 2015).

The success of the vitrification technique depends on different factors such as: the animal species, vitrification procedure (volume of the sample, cooling and warming rates) and sperm structure (Rosato and Iaffaldano, 2013). This study has been focused on the role of non-permeable CPAs and the equilibration temperatures, as key factors for stallion sperm vitrification.

Vitrification of sperm has been traditionally performed in different species at room temperature (Isachenko et al., 2008; Sanchez et al., 2011; Schulz et al., 2017). The stallion sperm, however, is sensitive to cold shock and the decrease of temperature from 19 to 8 °C is crucial (Moran et al., 1992). With results of the present study, the cooling period before vitrification is a key factor and the results obtained were much better than those obtained when vitrification was performed at room temperature. The progressive reduction of the temperature during the equilibration period at 5 °C may contribute to the acclimation of sperm membranes before freezing, facilitating membrane fluidity and, therefore, the interactions between additives and sperm membranes.

Sucrose has been widely used as a non-permeable agent for sperm vitrification (Isachenko et al., 2004b, 2008). The optimal

concentration of sucrose for sperm vitrification, however, depends on the species. Human (Isachenko et al., 2008) and dog (Sanchez et al., 2011) sperm need a concentration of sucrose of 250 mM while cat (Swanson et al., 2017), fish (Merino et al., 2012) and mouflon (Pradiee et al., 2017) require 200, 125, and 100 mM, respectively. Sperm of some of these species are extremely sensitive to the greater sucrose concentrations (Pradiee et al., 2017). In the present study, the upper limit to detect the harmful effect of sucrose concentration was 20 mM, which is far lower than those used previously, including a preliminary study of stallion sperm vitrification (Pérez-Marín et al., 2017) where concentrations from 125 to 250 mM of sucrose and trehalose were compared and there was very little sperm motility after warming. It appears as if stallion sperm are much more sensitive to chemical toxicity or osmotic shock induced by greater sucrose concentrations as compared with that in other species. Concentration of sucrose, therefore, is another key factor to consider in determining the success of stallion sperm vitrification. Further studies should be performed to ascertain potential beneficial effects of lesser concentrations of sucrose or other carbohydrates for stallion sperm vitrification.

Sucrose is usually combined with human or bovine serum albumin (BSA) for sperm vitrification (Isachenko et al., 2008; Sanchez et al., 2011; Merino et al., 2012; Diaz-Jimenez et al., 2017). The BSA functions to reduce reactive oxygen species (ROS) and thus oxidative stress (Uysal et al., 2005) and maintains the plasma membrane integrity due to prevention of lipid peroxidation and stabilization of membrane proteins (Cabrita et al., 2001; Uysal et al., 2005), providing energy to sperm and improving its survival after cryopreservation (Nang et al., 2012). Additionally, albumin functions as a bulking agent and increases the glass transition temperature of the extracellular matrix (Oldenhof et al., 2017b). There, however, is no information for the most suitable concentration in the vitrification media. On the contrary, in previous studies concentrations of BSA greater than 1% increase osmolality (Nang et al., 2012; Naijian et al., 2013), and are involved in capacitation and cholesterol efflux from sperm membranes, which could explain the deleterious effect on sperm cryopreservation. Nevertheless, with stallion sperm the effect of albumin on cholesterol incorporation in sperm membranes has not been observed (Macias-Garcia et al., 2015). The specific molecular mechanism by which BSA induces such harmful effects on sperm needs to be more precisely ascertained. In the present study the upper limit for BSA concentrations in stallion sperm vitrification appears to be 1%, which is consistent with findings in previous studies of sperm vitrification in other animal species (Sanchez et al., 2011; Merino et al., 2012; Pradiee et al., 2015).

Secondly, conventional slow freezing in nitrogen vapours was compared to vitrification with the selected media. The combination of 20 mM sucrose with 1% of BSA was optimal for stallion sperm vitrification being more effective than use of conventional freezing procedures. The addition of both albumin and sucrose contributed to cell dehydration before vitrification and increased the viscosity of the extracellular medium, maintaining the osmotic pressure of the diluents (Isachenko et al., 2017). Furthermore, there has been no evidence of ice crystal formation in frozen-thawed human and stallion sperm (Morris, 2006; Morris et al., 2007). It suggests that the cell damage during rapid cooling is due to an osmotic imbalance rather than the formation of intracellular ice. Vitrification using non-permeable agents, therefore, contributes to a more effective maintenance of the osmotic balance.

Cryodamage is mainly revealed as loss in membrane integrity after thawing (Sieme et al., 2016). In this regard, vitrification more effectively protected acrosome and plasma membrane integrity than conventional freezing, which is in contrast with previous studies in animal species (Jiménez-Rabadán et al., 2015; Arando et al., 2017; Pradiee et al., 2017). Additionally, conventional freezing obtained moderate results of sperm motility in the present study but similar to previous studies in stallions using different extenders (Medeiros et al., 2002; Scherzer et al., 2009; Wu et al., 2015). A commercial stallion semen extender was used in the present study for conventional freezing by adding glycerol as previously described (Scherzer et al., 2009; Álvarez et al., 2014). A number of components can be added to these extenders to improve post-thaw sperm variables such as low molecular weight CPAs (Wu et al., 2015), antioxidants (Chen et al., 1993) or egg-yolk (Salazar et al., 2011). These additives, particularly egg-yolk, could improve the results obtained in this study for conventional sperm freezing but egg yolk may also had a positive influence on vitrified sperm. To strictly compare vitrification and conventional freezing techniques using permeable and non-permeable CPAs, the base extender was exactly the same for both methods but replacing glycerol in conventional freezing with sucrose and BSA in sperm vitrification. Surprisingly, as previously described, vitrification of stallion sperm using this method was more effective in sperm cryopreservation than conventional freezing. The osmolality of the extenders containing glycerol is much greater than the alternatives used in the present study, which are similar to the iso-osmolality of the sperm. This difference in osmolality seems to be a major problem for sperm cryopreservation in stallions (Darr et al., 2016). Vitrification using non-permeable agents, therefore, avoids the damage to cells caused both by cryoprotectant toxicity and osmotic stress, which may help explain the results obtained in the present study.

Vitrification of sperm in small volumes has a direct application in use of assisted reproductive techniques in horses, particularly in ICSI where only a few number of sperm are needed (Canesin et al., 2017); or also in deep-horn artificial insemination, where 1–5 million sperm (Brinsko et al., 2003; Hayden et al., 2012) in a sample volume of 0.2–2 ml (Samper and Plough, 2010) has achieved acceptable pregnancy rates. Unfortunately, the application of this approach in commercial horse breeding programs using artificial insemination is limited. Further studies are needed focused on the development of other vitrification techniques using larger volumes of sperm for a more effective application of these technologies in the equine industry.

In conclusion, stallion sperm can be vitrified in small volumes (spheres) after equilibration/cooling at 5 °C using a combination of 20 mM sucrose and 1% BSA based extender obtaining better sperm parameters than sperm freezing with glycerol.

Conflict of interest

The authors declare no conflicts of interest.

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Original Research

Vitrification of Large Volumes of Stallion Sperm in Comparison With Spheres and Conventional Freezing: Effect of Warming Procedures and Sperm Selection

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ABSTRACT

Stallion sperm was vitrified using straws in comparison with spheres and conventional freezing. Vitrification was performed plunging 30 μL of sperm (spheres) or 0.5 mL straws into liquid nitrogen (LN_2) and conventional freezing using 0.5 mL straws frozen in LN_2 vapors. Sperm vitrified in straws were submitted to different warming procedures (42°C/20 seconds; 60°C/15 seconds) and single-layer centrifugation (SLC). Total (TM, %) and progressive sperm motility (PM, %), plasma membrane (IMS, %) and acrosome integrity (AIS, %) were statistically compared between treatments (mean ± SEM). Significant higher values ($P < .001$) were obtained after vitrification using spheres in comparison with conventional freezing and vitrification in straws for TM (54.46 ± 3.2 vs. 36.47 ± 3.2 vs. 2.50 ± 1.2, %), PM (38.63 ± 3.4 vs. 15.11 ± 2.0 vs. 1.9 ± 0.9, %), IMS (65.40 ± 2.8 vs. 50.50 ± 2.8 vs. 21.63 ± 2.1, %), and AIS (48.89 ± 2.8 vs. 15.46 ± 1.7 vs. 4.69 ± 0.9, %). No differences were found between warming procedures. Single-layer centrifugation after warming at 42°C/20 seconds obtained higher values ($P < .05$) than unselected samples for TM (32.52 ± 5.8%), PM (14.22 ± 2.8%), IMS (60.01 ± 3.2%), and AIS (44.5 ± 2.2%), whereas selection after 60°C/15 seconds increased TM (23.11 ± 4.3%) and IMS (67.11 ± 3.9%). In conclusion, vitrification in spheres obtained better sperm quality than conventional freezing and vitrification in straws. Warming procedures did not affect the sperm quality but SLC could be a strategy to enhance the quality of the samples after sperm vitrification using 0.5 mL straws.

1. Introduction

Kinetic vitrification of stallion semen has been successfully developed as an alternative method of cryopreservation. This vitrification method is based on high-speed cooling by dropping small volumes of semen into liquid nitrogen (LN_2) using permeable cryoprotectant-free extenders [1]. Vitrification of stallion semen following the spheres method has been recently proposed as a promising alternative to conventional sperm freezing [2]. However, this is a nonaseptic technique that requires low volumes of sperm (30 μL), which could be applied to some equine assisted reproductive technologies, such as intracytoplasmic sperm injection, but compromises current artificial insemination techniques with frozen semen [3]. Vitrification using straws allows the isolation of the semen from LN_2 (aseptic technique), reducing the potential risk

Animal welfare/ethical statement: This study was approved by the Ethical Committee for Animal Experimentation of the University of Cordoba, in compliance with the Regional Government of Andalusia (project no. 31/08/2017/105) and the Spanish law for animal welfare and experimentation (RD 53/2013 and Decision, 2012/707/UE).

Conflict of interest statement: None of the authors have any conflict of interest to declare.

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of microbial contamination [4] and increasing the sperm volume [5,6]. Recently, sperm vitrification of stallion, donkey, and human has been performed in 0.25 mL straws [7–9]. This method has been proposed as an alternative to conventional freezing and vitrification in spheres. Moreover, sperm vitrification using 0.5 mL straws has been accomplished in human sperm [5]; unfortunately, when this procedure was repeated in human, bovine, and stallion sperm, the results were unsatisfactory [10,11].

One of the most critical steps during sperm vitrification is the warming procedure, due to the risk of recrystallization during this process [12]. In previous studies of sperm vitrification, higher warming velocities (higher temperature and lower time of exposure) obtained better sperm motility and plasma membrane integrity than lower warming rates [13,14]. Different sperm selection techniques have usually been performed before vitrification [1,15], but not after warming of the vitrified samples. In conventional sperm freezing, the use of colloid single-layer centrifugation (SLC) has reported an improvement in equine semen quality after thawing [16]; therefore, different warming procedures and sperm selection could enhance the outcome of vitrification in large volumes, up to 0.5 mL.

The objectives of this study were to (1) vitrify stallion sperm in large volumes in comparison with vitrification in spheres, using conventional freezing as control and (2) assess the effect of different warming procedures and colloid SLC, to improve the sperm quality after vitrification of large volumes of stallion sperm.

2. Materials and Methods

This study was approved by the Ethical Committee for Animal Experimentation of the University of Cordoba, in compliance with the Regional Government of Andalusia (project no. 31/08/2017/105) and the Spanish law for animal welfare and experimentation (RD 53/2013). All chemical used were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise is stated. A milk-based extender formulated for equine semen was used for semen processing (INRA96; IMV Technologies, L'Aigle, France), adding glycerol (Carlos Erba Reagents, Barcelona, Spain) for conventional slow freezing [2] and sucrose or trehalose plus 1% of bovine serum albumin (BSA, ref. A7906) for sperm vitrification as described in the following.

2.1. Semen Collection and Processing

Semen was obtained from six clinically healthy stallions, five of them Purebred Spanish Horse, and one Hispano-Arab (aged 10–23 years), using a Missouri artificial vagina during the breeding season. Two to three ejaculates were collected from each animal twice a week obtaining a total number of 16 and 12 ejaculates for experiments 1 and 2, respectively. All the semen samples were evaluated before process as previously described by Ortiz et al. [17], and all had the following characteristics: gel-free volume >23 mL, sperm concentration >86.8 $\times$ 10^6 sperm/mL, total sperm motility (TM) >90.2% and progressive sperm motility (PM) >70.1%. Thereafter, semen was extended in the milk-based extender (1:1; v:v) and centrifuged at 600 *g*/10 minutes. The sperm pellets were resuspended to a final concentration of 50–100 $\times$ 10^6 sperm/mL and subjected to vitrification or conventional freezing as described in the following.

2.2. Vitrification and Warming of Straws

Sperm vitrification was performed in 0.5 mL straws according to Isachenko et al. [5], with some modifications. After centrifugation, sperm were diluted in the base extender adding 1% BSA and sucrose 20 mM (343 mOsm/kg) or trehalose 100 mM (465 mOsm/kg) for experiments 1 and 2, respectively. After that, samples were cooled for 1 hour at 5°C before vitrification. Then, 0.5 mL straws (CBS, Cryo Bio System, Paris, France) were filled with the sperm suspension, and both sides of the straw were heat-sealed with SYMS I sealer (Cryo Bio System) and plunged directly into LN_2. After no less than 24 hours of storage in LN_2 tanks, straws were warmed by immersion in a water bath at 42°C for 20 seconds (experiments 1 and 2) or at 60°C for 15 seconds before sperm analysis (experiment 2).

2.3. Vitrification and Warming of Spheres

Sperm vitrification was performed following the spheres method as previously described by Hidalgo et al. [2]. Samples were centrifuged at 600 *g*/10 minutes, and the sperm pellets were diluted in the base extender adding 20 mM of sucrose + 1% BSA. After cooling for 1 hour at 5°C, five 30 μL droplets of the diluted sperm were directly plunged into LN_2. When solidification occurred, the spheres were collected and packaged into 1.8 mL cryotubes. Warming was performed after at least 24 hours of storage in LN_2 tanks. Spheres were submerged one by one into 2 mL of the milk-based extender at 42°C. Afterward, sperm suspension was centrifuged (600 *g*/10 minutes) and the sperm pellet was resuspended in the same extender to a final concentration of 25 $\times$ 10^6 sperm/mL for sperm evaluation.

2.4. Conventional Freezing and Thawing

Semen samples were frozen following a standard protocol for stallions [18]. After centrifugation, sperm were diluted in the base extender adding 2.2% of glycerol (1,150 mOsm/kg) and then slowly cooled for 2 hours [2]. After that, 0.5 mL French plastic straws (Minitübe, Tiefenbach, Germany) were filled with the sperm suspension and frozen horizontally in racks placed 4 cm above the surface of LN_2 for 10 minutes. Then, straws were stored in LN_2 tanks. Straws were thawed by immersion in a water bath at 37°C for 30 seconds.

2.5. Colloid Single-Layer Centrifugation

Single-layer centrifugation was performed after sperm vitrification in straws as previously described by Johannisson et al. [19], with minor modifications. For each sample, 0.4 mL of extended sperm (50 $\times$ 10^6 sperm/mL) were layered on the top of 0.8 mL of Androcoll-E-Small (glycidoxypropyltrimethoxysilane-coated silica colloid) [20]. Then, the sperm suspension was centrifuged at 300 *g*

Table 1
Postwarming sperm parameters assessed in stallion sperm vitrified in straws and spheres or after conventional freezing and thawing.

Parameters	Vitrification in 0.5 mL Straws	Vitrification in Spheres	Conventional Freezing
TM (%)	2.50 ± 1.2^c	54.46 ± 3.2^a	36.47 ± 3.2^b
PM (%)	1.90 ± 0.9^c	38.63 ± 3.4^a	15.11 ± 2.0^b
IMS (%)	21.63 ± 2.1^c	65.40 ± 2.8^a	50.50 ± 2.8^b
AIS (%)	4.69 ± 0.9^c	48.89 ± 2.8^a	15.46 ± 1.7^b

TM, total motility; PM, progressive motility; IMS, plasma membrane integrity; AIS, acrosome-intact sperm.
Different superscripts indicate significant differences ($P < .001$). Values are expressed as mean ± SEM.

for 20 minutes. The supernatant was discarded, and the sperm pellet was resuspended in the milk-based extender to reach a concentration of 25×10^6 sperm/mL for sperm analysis. The yield of selected sperm was calculated according to the following formulae:

$$Yield(\%) = \frac{number\ of\ sperm\ in\ sperm\ pellet\ recovered\ after\ SLC}{number\ of\ sperm\ in\ initial\ load} \times 100$$

2.6. Postwarming Sperm Evaluation

Sperm motility was assessed using the Sperm Class Analyzer (SCA v5.4; Microptic S.L., Barcelona, Spain). The features of this system have been described previously [17]. Total (TM, %) and progressive sperm motility (PM, %) of sperm were recorded. Plasma membrane integrity (IMS, %) was assessed using Vital test kit (Halotech SL, Madrid, Spain) under fluorescence microscopy. The integrity of acrosome membrane (AIS, %) was assessed using the propidium iodide/peanut agglutinin–fluorescein isothiocyanate double stain [21].

2.7. Experimental Design

2.7.1. Experiment 1: Comparison of Sperm Vitrification Methods (Straws and Spheres) and Conventional Freezing

Sperm vitrification and conventional freezing were performed as described before. Thereafter, post-warming/thawing sperm parameters were assessed and compared among methods.

2.7.2. Experiment 2: Effect of Warming Procedures and Colloid Single-Layer Centrifugation in Vitrified Sperm Using Straws

First, the protocols for warming the straws were selected. Different temperatures (37°C, 42°C, and 60°C) and times of exposure of straws into the water bath (8, 10, 12, 15, 20, and 30 seconds) were assessed. For that purpose, the straws were filled with the vitrification media containing trehalose (without sperm) and were vitrified. The temperature reached in each extender after warming was measured with a digital thermometer (TP3001; Labbox S.L., Spain), using the conventional procedure for thawing 0.5 mL French straws (37°C/30 seconds) as control. After that, two warming protocols were chosen: 42°C/20 seconds (W42) and 60°C/15 seconds (W60). Second, SLC was performed after warming using both protocols, W42 and W60. Finally, postwarming sperm parameters were assessed and compared between warming procedures (W42 and W60), and selected or unselected samples (W42 SLC and W42; W60 SLC and W60).

2.8. Statistical Analysis

Results were expressed as mean ± standard error of the mean (SEM). Differences among treatments were analyzed using a mixed linear model (PROC MIXED) followed by the post hoc Duncan test with treatment as fixed factor and animals and ejaculates as random factors. All analyses were carried out with SAS v9.0 statistic package (Institute Inc, Cary, NC). The level of significance was set at $P < .05$.

3. Results

3.1. Experiment 1: Comparison of Sperm Vitrification Methods (Straws and Spheres) and Conventional Freezing

Results of experiment 1 are shown in Table 1. Sperm vitrification by the spheres method obtained the highest values ($P < .001$) for all the parameters assessed (TM = 54.46 ± 3.2%; PM = 38.63 ± 3.4%; IMS = 65.40 ± 2.8%; AIS = 48.89 ± 2.8%). By contrast, the lowest values were obtained using the straws method (TM = 2.50 ± 1.2%; PM = 1.90 ± 0.9%; IMS = 21.63 ± 2.1%; AIS = 4.69 ± 0.9%).

3.2. Experiment 2: Effect of Warming Procedures and Colloid Single-Layer Centrifugation in Vitrified Sperm Using Straws

Results of experiment 2 are shown in Table 2 and Fig. 1. The temperature reached in the samples after warming the straws with the control procedure (37°C/30 seconds) was 29.0 ± 0.4°C. The temperature reached in the samples warmed with the two selected protocols was 26.7 ± 0.2°C for W42 and 30.33 ± 0.3°C for W60 (Table 2). No significant differences were found between these warming protocols (W42 and W60) in any of the sperm parameters assessed (Fig. 1A). On the other hand, sperm selection using SLC after warming at W42 showed significant higher values ($P < .05$) than unselected samples for TM (32.52 ± 5.8% vs. 19.52 ± 3.5%), PM (14.22 ± 2.8% vs. 9.59 ± 1.9%), IMS (60.01 ± 3.2% vs. 42.59 ± 4.5%), and AIS (44.50 ± 2.2% vs. 33.25 ± 2.6%) (Fig. 1B). Single-layer centrifugation after W60 also obtained significant higher values ($P < .05$) than unselected samples for TM (23.11 ± 4.3% vs. 16.33 ± 3.1%) and IMS (67.11 ± 3.9% vs. 50.04 ± 4.5%) (Fig. 1C). The yield of selected sperm using SLC ranged from 8 to 56.5 % with an average value of 27.07 ± 2.83%.

4. Discussion

The main purpose of this study was to vitrify large volumes of stallion sperm following an aseptic technique, which isolates the sperm from LN_2. According to the devices available in the market, the 0.5 mL straw was selected for this purpose. This device is commonly used for conventional freezing and has been applied for

Table 2
Temperature achieved in the extender after warming the straws at different temperatures and times of exposure into the water bath.

Time (s)	Temperature of the Water Bath					
	37°C		42°C		60°C	
	Temperature Achieved in the Extender (°C)	Warming Rate (°C/s)	Temperature Achieved in the Extender (°C)	Warming Rate (°C/s)	Temperature Achieved in the Extender (°C)	Warming Rate (°C/s)
8	—	—	—	—	21.8 ± 0.4	—
10	—	—	16.7 ± 0.4	—	24.6 ± 0.3	1.6 ± 0.2
12	14.4 ± 0.3	—	20.1 ± 0.4	1.4 ± 0.4	27.7 ± 0.4	1.6 ± 0.3
15	18.6 ± 0.5	1.4 ± 0.8	23.7 ± 0.2	1.3 ± 0.1	30.3 ± 0.3	0.9 ± 0.1
20	24.5 ± 0.4	1.2 ± 0.9	26.7 ± 0.2	0.6 ± 0.1	33.3 ± 0.3	0.6 ± 0.1
30	29.0 ± 0.4	0.4 ± 0.3	—	—	—	—

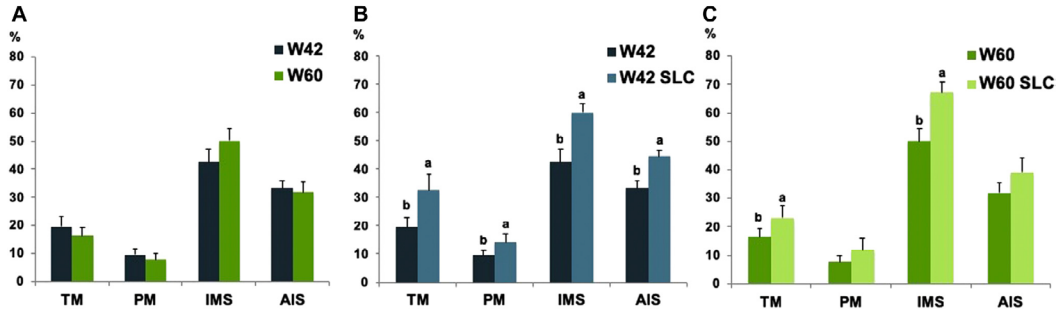

Fig. 1. Comparison of two warming protocols and sperm selection after vitrification using 0.5 mL straws: (A) W42 (42°C/20 seconds) and W60 (60°C/15 seconds); (B) W42 unselected and selected samples using single-layer centrifugation (W42 SLC); (C) W60 unselected and selected samples (W60 SLC). Different superscripts (a–b) indicate significant differences ($P < .05$). TM, total motility; PM, progressive motility; IMS, plasma membrane integrity; AIS, acrosome-intact sperm.

vitrification of large sperm volume in previous studies [5,6]. In the first experiment, sperm vitrification following the spheres method obtained better sperm quality after warming than conventional freezing, which is in agreement with previous studies in stallions and other species [2,22]. Unfortunately, the sperm quality achieved after vitrification and warming using 0.5 mL straws was much lower than the values obtained with conventional freezing or vitrification in spheres. Differences in the sperm quality between vitrification procedures could be due to differences in the volume or device used in each vitrification method [23,24]. Because of the poor sperm quality obtained after vitrification using the straws, the second experiment was performed to improve the sperm quality obtained with straws, changing the warming rate and selecting the most viable sperm.

One of the key factors for vitrification success is the selection of the extender, which varies not only between species [2,5,15] but also between vitrification protocols [2,7]. For this reason, in the second experiment, a different extender containing 100 mM of trehalose was used [25–27]. On the other hand, high warming temperatures included in the selected protocols (W42 and W60) have been used for vitrification and warming of human (42°C) [13] and mouflon sperm (60°C) [14], increasing the sperm quality by reducing the chance of recrystallization during warming [12]. Other temperatures from 70°C to 80°C have been also used for thawing 0.5 mL straws after conventional sperm freezing and resulted in higher sperm motility, integrity of plasma, and acrosome membranes than the lower temperatures assessed in the same study [28]. To the best of our knowledge, such high temperatures have never been used for warming vitrified straws and therefore could be considered in further studies.

Colloid SLC has been performed in stallions to select motile and viable sperm from samples with poor sperm quality [16,29]. In this study, SLC increased all the sperm parameters assessed after vitrification and warming, which is in agreement with previous studies in stallions and donkeys where SLC was performed after conventional freezing and thawing [16,30]. Nevertheless, the sperm quality obtained was not good enough to consider current techniques of sperm vitrification in 0.5 mL straws as an alternative to conventional freezing or vitrification in spheres in stallion sperm. Further studies are needed to assess other factors that could affect vitrification success in 0.5 mL straws.

In conclusion, stallion sperm vitrification in spheres obtained better sperm quality than conventional freezing and vitrification in straws. Warming procedures did not affect the sperm quality but SLC could be a strategy to enhance the quality of the samples after sperm vitrification using 0.5 mL straws.

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SHORT COMMUNICATION

WILEY Reproduction in Domestic Animals

Comparison of different sucrose-based extenders for stallion sperm vitrification in straws

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Contents

Vitrification of sperm is based on high-speed freezing by direct exposure to liquid nitrogen using non-permeable cryoprotectants, mainly disaccharides; yet, the concentration of cryoprotectants has a species-specific effect on the sperm cell. The aim of this study was to assess different sucrose concentrations for stallion sperm vitrification. Semen samples (n = 9) were collected from three stallions, centrifuged and resuspended to a concentration of 50 × 10^6 sperm/ml in a base extender (INRA96 + 1% of bovine serum albumin) with three different sucrose concentrations (Molar): 20 mM (S1), 100 mM (S2), or 200 mM (S3). Then, sperm were filled in covered 0.25 ml straws and directly plunged into liquid nitrogen. For warming, 0.25 ml straw was pulled out the covering straw and immersed in 3 ml of INRA96 at 43°C, with gentle pipetting to accelerate the melting. Total (TM, %) and progressive sperm motility (PM, %) were analysed using computer-assisted sperm analysis. Plasma (PMI, %) and acrosome membrane integrity (AIS, %) were assessed under epifluorescence microscopy. Post-warmed sperm parameters were compared between treatments by ANOVA. S2 showed significantly higher values in comparison with S1 and S3 for TM (S2 = 54.7 ± 5.5^a; S1 = 29.1 ± 3.3^b; S3 = 28.6 ± 3.0^b; $p < 0.001$) and PM (S2 = 31.3 ± 3.8^a; S1 = 18.5 ± 2.6^b; S3 = 17.7 ± 2.9^b; $p < 0.01$), respectively. No significant differences were found among treatments for PMI (S2 = 70.3 ± 5.2; S1 = 67.4 ± 4.3; S3 = 70.0 ± 3.7) neither for AIS (S2 = 57.1 ± 3.9; S1 = 53.9 ± 4.2; S3 = 57.0 ± 7.9). In conclusion, a concentration of 100 mM sucrose is recommended for stallion sperm vitrification in straws.

KEYWORDS
sperm, stallion, straw, sucrose, vitrification

1 | INTRODUCTION

Vitrification is an alternative method of cryopreservation, based on high-speed freezing and the use of high concentrations of cryoprotectants; yet, those concentrations of cryoprotectants have a toxic effect on sperm cell. Kinetic sperm vitrification was achieved by direct plunging of small volumes of sperm into liquid nitrogen (LN_2) without any permeable cryoprotectant, adding instead carbohydrates and proteins. The proper concentration of these cryoprotectants seems to be species specific (Isachenko et al., 2011; Pradiee et al., 2015; Sanchez et al., 2011) and depends on the volume of the sperm suspension (Arav et al., 2002).

Aseptic vitrification techniques involve the use of a packing system that allows the isolation of sperm from the LN_2 (Isachenko, Rahimi, Mallmann, Sanchez, & Isachenko, 2017). Recently, sperm vitrification in sterile straws have been successfully developed for human and other animal species (Daramola et al., 2016; Diaz-Jimenez et al., 2017; Isachenko et al., 2011), but not in horses. For

this purpose, the aim of this study was to assess different sucrose concentrations for stallion sperm vitrification without permeable cryoprotectants in straws in terms of post-warming sperm motility, plasma and acrosome membrane integrity.

2 | MATERIAL AND METHODS

All procedures have been approved by the Ethical Committee on Animal Experimentation of the University of Cordoba, in compliance with the Regional Government of Andalusia (project no. 31/08/2017/105) and the Spanish law for animal welfare and experimentation (RD 53/2013).

Semen samples (n = 9) were collected during the breading season from three healthy and fertile stallions (three ejaculates per animal) aged from 4 to 11 years, with a Missouri artificial vagina. Ejaculates were assessed for sperm volume, concentration, motility and plasma and acrosome membrane integrity as previously described (Consuegra et al., 2018). Then, sperm was diluted 1:1 (v:v) with INRA96 (IMV-Technologies, France), and centrifuged for 10 min at 600 *g*. The sperm pellets were resuspended to a final concentration of 50 × 10^6 sperm/ml in a base extender (INRA96 + 1% of bovine serum albumin [BSA]) adding sucrose at different concentrations: 20 mM (S1), 100 mM (S2), or 200 mM (S3), respectively. After that, extended samples were cooled for one hour at 5°C. Then, 0.25-ml French straws were filled with 100 µl of the sperm suspensions and inserted unsealed into 0.5 ml straws. After that, both extremes of each 0.5 ml straw were heat sealed with SYMS I sealer (Cryo Bio System, France) and plunged into LN_2. For warming, 0.25 ml straw was pulled out of the covering straw and immersed in 3 ml of INRA96 at 43°C with gentle pipetting until the vitrified solution became liquid (approximately 10 s). Post-thaw sperm suspension was centrifuged as described above, and the sperm pellet was resuspended in INRA96 to a final concentration of 25 × 10^6 sperm/ml for sperm evaluation. Total (TM, %) and progressive sperm motility (PM, %) were analysed using the Sperm Class Analyzer (SCA v.5.4 Microptic S.L., Spain). Plasma membrane integrity (PMI, %) was assessed using Vitaltest kit (Halotech DNA SL, Spain). Acrosome membrane integrity (AIS, %) was evaluated using propidium iodide/peanut agglutinin–fluorescein isothiocyanate double stain. Both plasma and acrosome membranes integrities were assessed under fluorescence microscopy (Olympus BX-40, Japan). Post-warmed sperm parameters were compared between treatments by ANOVA followed by Duncan test. Results were expressed as mean ± *SEM*. The level of significance was set at $p < 0.05$.

3 | RESULTS

Mean average parameters of ejaculates used in this study were as follows: gel-free volume = 53 ml; concentration = 197 × 10^6 sperm/ml; TM = 86%; PM = 70%; PMI = 92%; AIS = 83%.

The results of sperm parameters using vitrification are shown in Figure 1. Significant higher values were obtained with 100 mM sucrose (S2) in comparison to 20 mM (S1) and 200 mM (S3) for TM (S2 = 54.7 ± 5.5^a; S1 = 29.1 ± 3.3^b; S3 = 28.6 ± 3.0^b; $p < 0.001$) and PM (S2 = 31.3 ± 3.8^a; S1 = 18.5 ± 2.6^b; S3 = 17.7 ± 2.9^b; 0 < 0.01). No significant differences were found for PMI (S2 = 70.3 ± 5.2; S1 = 67.4 ± 4.3; S3 = 70.0 ± 3.7) neither for AIS (S2 = 57.1 ± 3.9; S1 = 53.9 ± 4.2; S3 = 57.0 ± 7.9).

4 | DISCUSSION

This is the first report of stallion semen vitrification successfully performed in straws using a combination of sucrose and BSA-based extenders. Vitrification in straws isolates sperm from LN_2, reducing the risk of microorganisms contamination (Isachenko et al., 2017), but also allows the vitrification of larger sperm volumes, increasing its application in reproductive technologies (Sanchez et al., 2012). A large number of studies have followed the sphere method for sperm vitrification but few of them used aseptic vitrification in straws. In this sense, human (Sanchez et al., 2012) and donkey (Diaz-Jimenez et al., 2017) sperm vitrification needs 250 mM of sucrose, which is higher than the best sucrose concentration obtained in this study (100 mM). It seems that stallion sperm is more sensitive than other species to the osmotic shock provoked by higher sucrose concentrations. Therefore, each species requires its own vitrification protocol, even those that are phylogenetically closely related (Pukazhenthi & Wildt, 2004).

The values of sperm parameters achieved in this study for stallion sperm vitrification were similar to previous results following conventional freezing protocols (Scherzer et al., 2009; Wu et al., 2015). Yet, no comparison between conventional freezing and vitrification has been performed in this study. Therefore, further studies are needed, including also the assessment of larger volumes and concentrations of sperm in different packing systems and extenders for a better application in the equine industry.

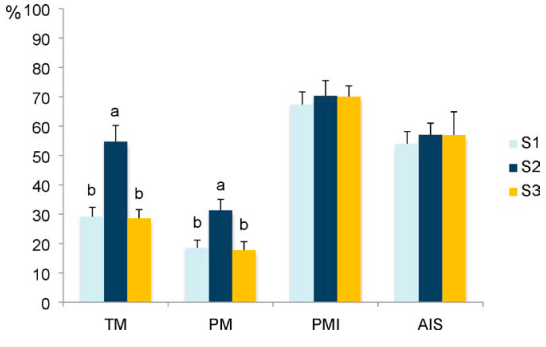

FIGURE 1 Total (TM) and progressive motility (PM), plasma (PMI) and acrosome membrane integrity (AIS) in stallion sperm after vitrification adding 1% BSA and 20 mM (S1), 100 mM (S2) or 200 mM (S3) of sucrose. Different letters indicate significant differences for TM ($p < 0.001$) and PM ($p < 0.01$)

In conclusion, stallion sperm can be vitrified in 0.25 ml straws using a combination of 100 mM sucrose and 1% BSA, without the addition of permeable cryoprotectants.

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CONFLICT OF INTEREST

None of the authors have any conflict of interest to declare.

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CHAPTER 1
Stallion sperm freezing with sucrose extenders: A strategy to avoid permeable cryoprotectants
Consuegra et al., 2018. Animal Reproduction Science

CHAPTER 2
Concentrations of non-permeable cryoprotectants and equilibration temperatures are key factors for stallion sperm vitrification success
Hidalgo et al., 2018. Animal Reproduction Science

CHAPTER 3
Vitrification of large volumes of stallion sperm in comparison to spheres and conventional freezing: effect of warming procedures and sperm selection
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CHAPTER 4

Chapter 4.1
Comparison of different sucrose-based extenders for stallion sperm vitrification in straws
Consuegra et al., 2018. Reproduction in Domestic Animals

Chapter 4.2
Vitrification of stallion sperm using 0.25 mL straws: Effect of volume, concentration and carbohydrates (sucrose/trehalose/raffinose)
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CHAPTER 5
Fertilizing capacity of vitrified stallion sperm utilizing heterologous IVF after different semen warming procedure
Consuegra et al., 2020. Animal Reproduction Science

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Vitrification of stallion sperm using 0.25 ml straws: Effect of volume, concentration and carbohydrates (sucrose/trehalose/raffinose)

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ABSTRACT

Sperm vitrification is a rapid freezing method in which carbohydrates are used as cryoprotectants. The aim of this study was to determine the optimal volume, concentration and type of carbohydrates for stallion sperm vitrification using 0.25 ml straws in comparison to conventional freezing. Ejaculates ($n = 54$) were collected from six stallions. For vitrification, straws were filled with different volumes (30, 70, 100 µl), sperm concentrations (50, 100, 200 × 10^6 sperm/ml) and extenders containing sucrose (20, 100, 200 mM), trehalose (50, 100, 200 mM) and raffinose (50, 100, 200 mM) and plunged into LN_2. Conventional freezing was performed in 0.5 ml straws frozen in LN_2 vapors. Sperm motility, plasma and acrosome membrane integrities and DNA fragmentation were compared among treatments. The use of straws filled with 100 µl at 100 × 10^6 sperm/ml with the extender containing 100 mM trehalose resulted in greater values for sperm quality than the other concentrations, volumes and carbohydrates. With vitrification, there were greater values (mean ± SEM; $P < 0.05$) than freezing for progressive motility (48.2 ± 2.3 compared with 37.3 ± 2.2%), plasma membrane integrity (82.8 ± 1.5 compared with 74.1 ± 1.9%), and intact acrosomes (50.2 ± 1.2 compared with 43.1 ± 1.4%); and less DNA fragmentation (6.4 ± 0.7 compared with 8.2 ± 0.3%). In conclusion, stallion sperm can be vitrified in 0.25 ml straws filled with 100 µl of sperm at 100 x 10^6 sperm/ml using an extender with 100 mM of trehalose, obtaining better sperm quality after warming than conventional freezing.

1. Introduction

Cryopreservation is used in the horse industry for long-term storage of stallion sperm. In general, slow cooling rates and extenders containing permeable cryoprotectants are used for storage of stallion semen. This procedure results in an osmotic imbalance for sperm because of the use of these processes as well as from the addition of cryoprotectants (Peña et al., 2011). Sperm vitrification is an alternative method of cryopreservation, performed by plunging small volumes of semen directly into liquid nitrogen (LN_2), without using any permeable cryoprotectant. Nevertheless, carbohydrates and proteins are added as non-permeable agents

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(Isachenko et al., 2017).

There are important factors for vitrification success, such as the semen volume, vitrification media or the equilibration period (Arav et al., 2002; Hidalgo et al., 2018). In previous studies of different species, including stallions, vitrification has been conducted with success using the spheres method (Isachenko et al., 2008; Sanchez et al., 2011; Pradiee et al., 2015; Hidalgo et al., 2018). This, however, is a non-aseptic technique, where a smaller volume of semen is utilized, and as a result, compromises the procedures used for artificial insemination of mares (Govaere et al., 2014). The straw method has been used as an alternative to this technique. This procedure provides for the opportunity to use a larger volume and concentration of sperm as well as for isolating sperm from LN_2, avoiding the risk of cross-contamination (Isachenko et al., 2017). The optimal volume and sperm concentration for stallion sperm vitrification using straws have yet to be determined. The addition of carbohydrates to the extender results in a greater viscosity of the solution that suppresses ice crystal formation, therefore, enhancing the vitrification process (Arav et al., 2002). For this purpose, sucrose has been the carbohydrate most commonly used for sperm vitrification in different species (Isachenko et al., 2008; Sanchez et al., 2011; Bóveda et al., 2018). Recently, other carbohydrates, such as trehalose and raffinose, have been also evaluated for sperm vitrification (Horta et al., 2017; Schulz et al., 2017; Caturla-Sánchez et al., 2018). The optimal concentration of each sugar appears to be species-specific. In this regard, Iberian ibex sperm (Pradiee et al., 2015) appear to be more sensitive to relatively greater concentration of carbohydrates than dog (Caturla-Sánchez et al., 2018) or donkey (Diaz-Jimenez et al., 2017, 2019) sperm. This fact is particularly relevant when considering cryopreservation of stallion sperm, which have previously been found to have poor resistance to the osmotic stress when there is greater molarity of carbohydrates, not only for conventional freezing (Consuegra et al., 2018a) but also for vitrification (Hidalgo et al., 2018).

The aim of the present study is to evaluate the effect of sperm concentration and volume as well as use of different molarities of sucrose, trehalose and raffinose, for stallion sperm vitrification in 0.25 ml straws as an alternative to conventional freezing.

2. Materials and methods

This study was approved by the Ethical Committee for Animal Experimentation of the University of Cordoba, in compliance with the Regional Government of Andalusia (project no. 31/08/2017/105) and the Spanish law for animal welfare and experimentation (RD 53/2013).

All chemicals used were purchased from Sigma Aldrich (Sigma-Aldrich, USA) unless stated otherwise. The basic medium used for sperm processing was a commercial milk fraction-based extender (INRA96, IMV Technologies, France) added to bovine serum albumin (BSA) and with inclusion of different concentrations of carbohydrates for sperm vitrification.

2.1. Animals

Six stallions of different breeds (five Purebred Spanish Horse and one Hispano-Arab) aged from 10 to 23 years, clinically healthy and with known fertility (> 60%) were used as semen donors. Animals were housed in individual paddocks placed at the Equine Breeding Centre of the Spanish Army located in Avila, Spain (40.66 °N, 4.70 °W).

2.2. Semen collection and processing

Semen samples were obtained using a Missouri-model artificial vagina in the presence of a mare in estrus. Semen was collected during the breeding season once or twice a week with nine ejaculates being collected from each stallion ($n = 54$). In each experiment, three ejaculates per animal were used ($n = 18$). Sperm quality of ejaculates was evaluated before freezing and had physiological values (mean ± SEM) for: gel-free volume (49.5 ± 2.9 ml), measured in a graduated collector, sperm concentration (269.2 ± 10.8 × 10^6 sperm/ml), assessed with a sperm photometer (Spermacue, Minitube, Germany) and total sperm motility (95.8 ± 0.5%), progressive sperm motility (78.6 ± 0.8%) and plasma membrane integrity (93.5 ± 0.6%), evaluated as subsequently described in this manuscript. Fresh semen was diluted with the milk-based extender at 37°C (1:1/v:v) and centrifuged for 10 min at 600 x *g*. The sperm pellets were re-suspended in different extenders for sperm vitrification or conventional freezing procedures (see experimental design). Osmolalities of all extenders were assessed using a freezing-point digital micro osmometer Type 6 (Löser Messtechnik, Germany).

2.3. Vitrification and warming of sperm

Sperm vitrification was conducted in covered 0.25 ml straws (Consuegra et al., 2018b). The semen samples diluted in the vitrification media were cooled for 1 h at 5°C. After cooling, 0.25 ml French straws were filled with the semen samples using a micropipette, and horizontally inserted in 0.5 ml straws. Subsequently, both ends of the external straw were heat-sealed and horizontally immersed in a styrofoam box containing liquid nitrogen (LN_2). Post-warming analysis was performed after 24 h of storage in LN_2 containers. For warming, 0.25 ml straw was removed from the covering straw and immersed in 3 ml of INRA96 pre-warmed to 43°C with gentle pipetting until the vitrified solution was liquid. The post-thaw sperm suspension was centrifuged (600 x *g* for 10 min) and the sperm pellet was re-suspended in milk-based extender to a final concentration of 25 × 10^6 sperm/ml for sperm evaluation.

2.4. Conventional sperm freezing and thawing

Semen samples were frozen using a standard protocol for stallions with modifications (Consuegra et al., 2018a). Briefly, centrifuged sperm pellets were re-suspended in a freezing extender containing 4% methylformamide and 1% glycerol (Botucrio, Nidacon, Sweden; osmolality = 1162 mOsm/kg) (Macedo et al., 2018), to a final concentration of 50×10^6 sperm/ml (Consuegra et al., 2018a). After that, samples were cooled to 5°C within 2 h, samples were then loaded into 0.5 ml straws (Ortiz et al., 2014). The straws were placed horizontally for freezing in racks that were positioned 4 cm above the surface of LN_2 for 10 min and plunged in LN_2, and subsequently then stored in LN_2 tanks for at least 24 h before further assessment occurred. Straws were thawed using the immersion procedure in a 37°C water bath for 30 s and re-suspended with the basic medium for semen analysis (INRA96).

2.5. Post-thawing/warmed sperm evaluation

Sperm motility was objectively evaluated by CASA system (SCA v5.01, Microptic S.L., Spain). The features of this system have been described previously (Ortiz et al., 2014). The sperm kinematic variables recorded were: total (TM, %) and progressive motility (PM, %), curvilinear (VCL, μm/s), straight line (VSL, μm/s) and average path velocities (VAP, μm/s), linearity (LIN, VSL/VCLx100), straightness (STR, VSL/VAPx100), wobble (WOB, VAP/VCLx100), amplitude of lateral head displacement (ALH, μm) and beat cross frequency (BCF, Hz). Sperm motility was considered progressive when VCL > 90 μm/s and STR > 75%.

The integrity of plasma membrane was assessed utilizing the Vital test (Halotech SL, Spain) using fluorescence microscopy as previously described (Cortés-Gutiérrez et al., 2008). The percentage of sperm with intact plasma membrane was recorded (IMS, %). Acrosome integrity was evaluated using the propidium iodide/peanut agglutinin–fluorescein isothiocyanate double stain (Dorado et al., 2014). At least 200 sperm were evaluated on each slide. Percentages of sperm with an intact acrosome membrane (AIS, %) were recorded.

Sperm DNA integrity was assessed using the Sperm-Halomax kit (Halotech SL, Madrid, Spain) for stallion sperm (López-Fernández et al., 2007) and a commercial DNA stain using fluorescence microscopy (Fluogreen, Halotech SL, Madrid, Spain) following the manufacturer's instructions. At least 200 sperm per sample were evaluated. Sperm with a large halo of chromatin dispersion were recorded as sperm with fragmented DNA (sDF, %). Sperm DNA fragmentation was assessed after 0, 4, 8 and 24 h of incubation at 37°C after thawing/warming.

2.6. Experimental design

2.6.1. Experiment 1. Evaluation of different volumes and concentrations for sperm vitrification using straws

After semen processing, sperm were extended in a milk-based extender adding sucrose at 20 mM (mmol/l) plus 1% BSA (314 mOsm/kg) with the result being three different sperm concentrations: 50, 100 and 200 ($x10^6$ sperm/ml). Subsequently, the 0.25 ml straws covered with 0.5 ml straws were filled with sperm at each concentration but using different volumes: 30, 70 and 100 μl. Both ends of 0.5 ml straws were subsequently heat-sealed as previously described. The sperm quality variables were assessed following warming and comparisons were made among values obtained using different volumes and concentrations.

2.6.2. Experiment 2. Comparison among sucrose, trehalose and raffinose for sperm vitrification in straws

The results in Experiment 1 from assessing the combination of semen volume and sperm concentration resulting in the greatest values for sperm quality variables were used to determine the procedures performed in Experiment 2. These determinations resulted in using straws filled with 100 μl at 100×10^6 sperm/ml in subsequent experiments. Semen samples were diluted in the base extender with addition of three different carbohydrates occurring at three different molarities as follows: sucrose 20 mM (S1; 314 mOsm/kg) (Hidalgo et al., 2018), 100 mM (S2; 454 mOsm/kg) and 200 mM (S3; 591 mOsm/kg); trehalose 50 mM (T1; 394 mOsm/kg) (Schulz et al., 2017), 100 mM (T2; 465 mOsm/kg) and 200 mM (T3; 587 mOsm/kg) of; and raffinose 50 mM (R1; 382 mOsm/kg), 100 mM (R2; 449 mOsm/kg) and 200 mM (R3; 552 mOsm/kg). The values for post-warming sperm variables were assessed and compared among treatments.

2.6.3. Experiment 3. Evaluation of sperm vitrification in straws in comparison to conventional freezing

The vitrification treatment resulting in the greatest sperm quality in Experiment 2 (T2 = 100 Mm trehalose) was selected and compared with those obtained with conventional freezing. Semen samples were divided into two aliquots: one was frozen using the conventional freezing protocol; the other was vitrified using the extender with 100 mM of trehalose to a final concentration of 100×10^6 sperm/ml, filling the 0.25 ml straws with 100 μl of the sperm suspension and then covered with the 0.5 ml straw. Subsequently, the values for post-thawing sperm variables were assessed and compared among treatments.

2.7. Statistical analysis

An analysis of the data was conducted using SAS v9.0 (SAS Institute Inc., NC, USA). A general linear model (PROC GLM) and the Duncan test with animals and ejaculates as random factors, were performed for the analysis. For each sperm variable, normality and homogeneity of variances were assessed using the Kolmogorov-Smirnov and Levene test, respectively. A linear regression analysis was conducted for values of DNA fragmentation at different incubation times and the slopes (sDF%/hour) were compared among treatments using the GraphPad Prism v.6 for Mac OS (GraphPad Software, CA, USA). Results were expressed as mean ± standard error of the mean (SEM). The level of significance was set at $P < 0.05$.

Table 1
Comparison of different volumes and concentrations (m = million) for stallion sperm vitrification in 0.25 ml straws.

Variables	Sperm vitrification procedures									
	30 µl/50 m	70 µl/50 m	100 µl/50 m	30 µl/100 m	70 µl/100 m	100 µl/100 m	30 µl/200 m	70 µl/200 m	100 µl/200 m	*P*-value
TM (%)	31.6 ± 2.6^{c}	41.2 ± 3.9^{abc}	47.1 ± 4.0^{ab}	40.0 ± 4.2^{abc}	40.5 ± 3.9^{abc}	49.8 ± 3.4^{a}	36.0 ± 3.5^{bc}	40.5 ± 3.2^{abc}	47.2 ± 4.2^{ab}	< 0.05
PM (%)	17.9 ± 2.9^{c}	24.2 ± 3.1^{abc}	27.6 ± 3.4^{ab}	19.3 ± 2.5^{bc}	22.4 ± 2.8^{abc}	31.1 ± 2.7^{a}	17.8 ± 1.9^{c}	24.4 ± 2.3^{abc}	29.4 ± 3.4^{a}	< 0.01
IMS (%)	63.0 ± 2.8^{a}	67.0 ± 2.4^{a}	70.8 ± 2.6^{a}	64.5 ± 2.9^{a}	65.6 ± 2.4^{a}	66.5 ± 2.4^{a}	66.1 ± 2.2^{a}	67.2 ± 2.2^{a}	68.1 ± 2.4^{a}	> 0.05
AIS (%)	39.2 ± 3.3^{a}	46.2 ± 2.5^{a}	48.7 ± 2.9^{a}	44.9 ± 2.1^{a}	44.3 ± 2.8^{a}	49.2 ± 2.5^{a}	44.4 ± 2.5^{a}	48.5 ± 2.8^{a}	49.7 ± 2.7^{a}	> 0.05
VCL (µm/s)	45.9 ± 2.6^{b}	52.1 ± 4.8^{ab}	53.3 ± 2.9^{ab}	46.8 ± 1.7^{b}	48.9 ± 2.9^{b}	59.6 ± 3.4^{a}	47.6 ± 1.7^{b}	55.3 ± 3.2^{ab}	52.9 ± 1.9^{ab}	< 0.05
VSL (µm/s)	31.2 ± 1.7^{c}	35.5 ± 2.3^{abc}	39.3 ± 2.0^{ab}	33.1 ± 1.4^{bc}	36.1 ± 2.6^{abc}	41.9 ± 2.3^{a}	33.6 ± 1.4^{bc}	39.1 ± 2.4^{ab}	38.4 ± 2.1^{ab}	< 0.01
VAP (µm/s)	35.0 ± 2.2^{c}	40.1 ± 2.9^{abc}	43.6 ± 2.4^{ab}	37.3 ± 1.5^{bc}	39.3 ± 2.7^{abc}	46.6 ± 2.6^{a}	37.3 ± 1.4^{bc}	43.4 ± 2.8^{ab}	42.0 ± 2.1^{abc}	< 0.05
LIN (%)	68.7 ± 2.4^{a}	71.1 ± 3.2^{a}	74.1 ± 1.7^{a}	71.0 ± 2.2^{a}	68.9 ± 4.4^{a}	70.4 ± 2.1^{a}	70.7 ± 1.9^{a}	70.5 ± 2.1^{a}	71.7 ± 2.0^{a}	> 0.05
STR (%)	89.8 ± 1.4^{a}	89.2 ± 1.9^{a}	90.3 ± 0.7^{a}	88.9 ± 1.8^{a}	90.6 ± 1.1^{a}	89.7 ± 0.8^{a}	90.1 ± 1.6^{a}	90.0 ± 1.2^{a}	90.8 ± 0.8^{a}	> 0.05
WOB (%)	76.2 ± 2.0^{a}	79.1 ± 2.6^{a}	82.0 ± 1.6^{a}	79.6 ± 1.4^{a}	80.5 ± 1.9^{a}	78.4 ± 1.9^{a}	78.5 ± 1.4^{a}	78.2 ± 1.8^{a}	78.8 ± 1.8^{a}	> 0.05
ALH (µm)	2.3 ± 0.1^{a}	2.4 ± 0.1^{a}	2.4 ± 0.1^{a}	2.5 ± 0.1^{a}	2.4 ± 0.1^{a}	2.6 ± 0.1^{a}	2.5 ± 0.1^{a}	2.6 ± 0.1^{a}	2.7 ± 0.1^{a}	> 0.05
BCF (Hz)	7.0 ± 0.5^{a}	7.2 ± 0.5^{a}	6.6 ± 0.3^{a}	6.9 ± 0.2^{a}	6.8 ± 0.3^{a}	7.3 ± 0.5^{a}	7.2 ± 0.5^{a}	7.3 ± 0.3^{a}	7.0 ± 0.3^{a}	> 0.05

TM= total motility; PM = progressive motility; IMS = plasma membrane integrity; AIS = acrosome-intact sperm; VCL= curvilinear velocity; VSL = linear velocity; VAP = average path velocity; LIN = linear coefficient; STR = straightness coefficient; WOB = Wobble coefficient; ALH = lateral head displacement; BCF = beat cross frequency. Different superscripts (a, b, c) indicate significant differences ($P < 0.05$); Values are expressed as mean ± SEM.

3. Results

3.1. Experiment 1. Evaluation of different volumes and concentrations of sperm for vitrification in straws

The lowest values ($P < 0.01$) for PM, VCL, VSL and VAP were obtained using a volume of 30 μl at any sperm concentration. There were no differences for IMS, AIS, LIN, STR, WOB, ALH and BCF among the volumes and concentrations assessed. When vitrification was performed in straws filled with 100 μl at 100 x 10^6 sperm/ml, values for TM, PM, VCL, VSL and VAP were greater in comparison to values of the other treatment groups (Table 1); therefore, this procedure was selected for use in the subsequent experiments.

3.2. Experiment 2. Comparison among sucrose, trehalose and raffinose for sperm vitrification in straws

There were greater values for PM and IMS using a concentration of 100 mM of trehalose in comparison to 200 mM sucrose and 100 mM or 200 mM raffinose ($P < 0.05$). With respect to AIS, when 50 and 100 mM of trehalose were used, there were greater values ($P < 0.05$) in comparison to raffinose at any of the concentrations assessed ($P < 0.05$). There were no differences in values among treatment groups for the other variables that were assessed (Table 2). A concentration of 100 mM trehalose was, therefore, selected for the subsequent experiments.

3.3. Experiment 3. Evaluation of sperm vitrification in straws in comparison to conventional freezing

With vitrification using 100 mM of trehalose, there were greater values ($P < 0.05$) than with conventional freezing for PM (48.2 ± 2.3 compared with 37.3 ± 2.2%), IMS (82.8 ± 1.5 compared with 74.1 ± 1.9%), AIS (50.2 ± 1.2 compared with 43.1 ± 1.4%) and most of the values for other kinetic variables assessed (Fig. 1). Additionally, values for sDF at 0 h were greater ($P < 0.05$) with use of conventional freezing (8.2 ± 0.3%) in comparison to vitrification (6.4 ± 0.7%) (Fig. 2). There were no differences in the slopes when sDF values were assessed among treatment groups (Fig. 3).

4. Discussion

Kinetic sperm vitrification performed in straws is a relatively recently developed procedure (Sanchez et al., 2012). In the present study, the smallest French straws available for sperm freezing, 0.25 ml, were used. The straws were filled with different semen volumes ranging from 30 μl, which was previously used for stallion sperm vitrification in spheres (Hidalgo et al., 2018), to 100 μl, volume used in other species for sperm vitrification in 0.25 ml straws (Diaz-Jimenez et al., 2017; Schulz et al., 2017). The lesser volume was applied successfully using the spheres method in dog, human and stallion sperm vitrification (Isachenko et al., 2008; Sanchez et al., 2011; Hidalgo et al., 2018), so the use of this procedure was expected to result in a greater sperm quality among the volumes evaluated in the present study. The use of the greatest volume assessed (100 μl), however, resulted in the greatest total and progressive sperm motility. This could be attributed to differences between methods (spheres compared with straws). When straws are used instead of spheres, there is a larger surface area of sperm exposed to LN_2, resulting in a more rapid cooling rate, as previously reported by Diaz-Jimenez et al. (2018). In this regard, it would be interesting to perform further studies using greater volumes of sperm for vitrification, because straws containing 0.25 ml can be filled with more than 100 μl.

Vitrification has been traditionally performed with human sperm using samples with small concentrations of sperm (Schulz et al., 2017). Semen doses used for regular artificial insemination techniques, however, need to contain spermatozoa in greater sperm concentrations (Sieme et al., 2004). In this regard, previous studies of sperm vitrification in different species have included relatively greater concentrations of sperm (Pradiee et al., 2015; Diaz-Jimenez et al., 2017). Unfortunately, there is no consensus as to what the optimal sperm concentration is for vitrification in animal species, varying from 2 to 200 × 10^6 sperm/ml (Sanchez et al., 2011; Diaz-Jimenez et al., 2017). In the present study, the sperm concentrations used ranged from 50 × 10^6 sperm/ml, a concentration which was previously used for stallion sperm vitrification in spheres (Hidalgo et al., 2018) to 200 × 10^6 sperm/m, that was previously used for donkey sperm vitrification in straws (Diaz-Jimenez et al., 2017). Based on results from the present study, the use of the intermediate concentration of 100 × 10^6 sperm/ml, resulted in the greatest motility values after warming. This finding is consistent with previous findings for sperm freezing where intermediate concentrations were recommended [26]. It has been proposed that cryopreservation using relatively greater concentrations of sperm (200 and 400 × 10^6 sperm/ml) could result in a reduction in sperm motility and viability (Nascimento et al., 2008). Additionally, lesser sperm concentrations could be associated with the deleterious "dilution effect" (Hayden et al., 2015). Either of these factors could result in the loss of motility observed in the sperm samples vitrified at relatively lesser and greater sperm concentrations.

In the present study, three carbohydrates were used for vitrification: sucrose, trehalose and raffinose. Sucrose has been the carbohydrate primarily used for sperm vitrification (Isachenko et al., 2008) due to its effectiveness in increasing the viscosity of the extracellular medium and stabilizing the sperm plasma membrane (Chen et al., 1993). In addition, trehalose and raffinose can trap the free radicals that lead to the peroxidation of the lipids of the plasma membrane, maintaining its integrity (Aisen et al., 2005; Bucak et al., 2013). Trehalose also enhances the fluidity of the sperm membrane and preserves the lipid bilayer by stabilization of the water structure around the membrane, protecting the sperm against cryodamage (Aboagla and Terada, 2003; Gheller et al., 2019). The concentrations of these carbohydrates are key factors for vitrification success (Isachenko et al., 2003; Hidalgo et al., 2018). Sperm of some species are more sensitive to the osmotic stress caused by relatively greater carbohydrate concentrations than sperm of

Table 2
Comparison of different molarities of non-permeable cryoprotectants (sucrose, trehalose and raffinose) for stallion sperm vitrification in 0.25 ml straws.

Variables	Sperm vitrification treatments (carbohydrates)									
	S1	S2	S3	T1	T2	T3	R1	R2	R3	
	20 mM sucrose	100 mM sucrose	200 mM sucrose	50 mM trehalose	100 mM trehalose	200 mM trehalose	50 mM raffinose	100 mM raffinose	200 mM raffinose	*P*-Value
TM (%)	52.1 ± 3.7^{ab}	52.8 ± 3.3^{ab}	49.2 ± 3.1^{b}	56.3 ± 3.8^{ab}	60.3 ± 3.0^{a}	55.4 ± 2.6^{ab}	51.5 ± 3.3^{ab}	45.8 ± 3.0^{b}	47.9 ± 3.5^{b}	> 0.05
PM (%)	31.6 ± 2.2^{bc}	34.6 ± 2.9^{abc}	28.7 ± 2.6^{c}	39.1 ± 3.6^{ab}	41.5 ± 3.3^{a}	35.8 ± 9.0^{abc}	32.5 ± 2.3^{bc}	28.6 ± 2.2^{c}	30.5 ± 3.1^{bc}	< 0.01
IMS (%)	75.6 ± 2.3^{abc}	79.2 ± 1.7^{ab}	74.4 ± 2.2^{bc}	77.0 ± 2.3^{abc}	81.1 ± 2.1^{a}	78.4 ± 1.7^{ab}	73.3 ± 2.0^{bc}	72.9 ± 2.0^{bc}	71.2 ± 2.4^{c}	< 0.05
AIS (%)	47.1 ± 2.8^{ab}	49.1 ± 2.0^{ab}	45.5 ± 2.8^{ab}	51.6 ± 2.0^{a}	53.3 ± 2.0^{a}	47.3 ± 2.8^{ab}	41.2 ± 2.3^{b}	43.4 ± 2.4^{b}	43.2 ± 2.9^{b}	= 0.01
VCL (μm/s)	52.1 ± 2.0^{a}	54.3 ± 1.9^{a}	52.5 ± 2.4^{a}	56.4 ± 1.9^{a}	55.4 ± 2.0^{a}	54.3 ± 1.6^{a}	52.4 ± 1.6^{a}	53.5 ± 2.2^{a}	53.2 ± 2.6^{a}	> 0.05
VSL (μm/s)	39.5 ± 1.7^{a}	42.1 ± 2.0^{a}	37.5 ± 1.9^{a}	43.5 ± 1.9^{a}	43.1 ± 1.8^{a}	42.0 ± 1.6^{a}	40.5 ± 1.5^{a}	40.0 ± 1.7^{a}	39.8 ± 1.7^{a}	> 0.05
VAP (μm/s)	42.9 ± 1.9^{a}	45.6 ± 2.1^{a}	42.7 ± 2.2^{a}	47.1 ± 2.0^{a}	46.9 ± 1.9^{a}	45.9 ± 1.6^{a}	43.5 ± 1.5^{a}	44.0 ± 2.2^{a}	44.1 ± 2.2^{a}	> 0.05
LIN (%)	75.6 ± 1.5^{a}	77.3 ± 2.0^{a}	72.7 ± 2.0^{a}	76.9 ± 1.8^{a}	77.7 ± 1.7^{a}	77.2 ± 1.3^{a}	77.2 ± 1.4^{a}	75.0 ± 1.6^{a}	75.6 ± 2.1^{a}	> 0.05
STR (%)	92.0 ± 0.6^{a}	92.2 ± 0.7^{a}	89.3 ± 0.9^{a}	91.9 ± 0.7^{a}	91.9 ± 0.7^{a}	91.4 ± 0.6^{a}	92.8 ± 0.6^{a}	91.3 ± 1.1^{a}	90.9 ± 1.2^{a}	> 0.05
WOB (%)	82.1 ± 1.3^{a}	83.7 ± 1.6^{a}	81.3 ± 1.7^{a}	83.5 ± 1.5^{a}	84.5 ± 1.4^{a}	84.3 ± 1.0^{a}	83.2 ± 1.2^{a}	82.1 ± 1.4^{a}	83.0 ± 1.5^{a}	> 0.05
ALH (μm)	2.5 ± 0.1^{a}	2.4 ± 0.1^{a}	2.6 ± 0.2^{a}	2.5 ± 0.1^{a}	2.4 ± 0.1^{a}	2.4 ± 0.1^{a}	2.4 ± 0.1^{a}	2.5 ± 0.1^{a}	2.4 ± 0.1^{a}	> 0.05
BCF (Hz)	7.0 ± 0.2^{a}	6.9 ± 0.2^{a}	6.9 ± 0.2^{a}	7.1 ± 0.2^{a}	6.8 ± 0.1^{a}	6.8 ± 0.1^{a}	7.1 ± 0.1^{a}	7.2 ± 0.2^{a}	6.7 ± 0.1^{a}	> 0.05

TM = total motility; PM = progressive motility; IMS = plasma membrane integrity; AIS = acrosome-intact sperm; VCL = curvilinear velocity; VSL = linear velocity; VAP = average path velocity; LIN = linear coefficient; STR = straightness coefficient; WOB = Wobble coefficient; ALH = lateral head displacement; BCF = beat cross frequency. Different superscripts (a, b, c) indicate significant differences ($P < 0.05$); Values are expressed as mean ± SEM.

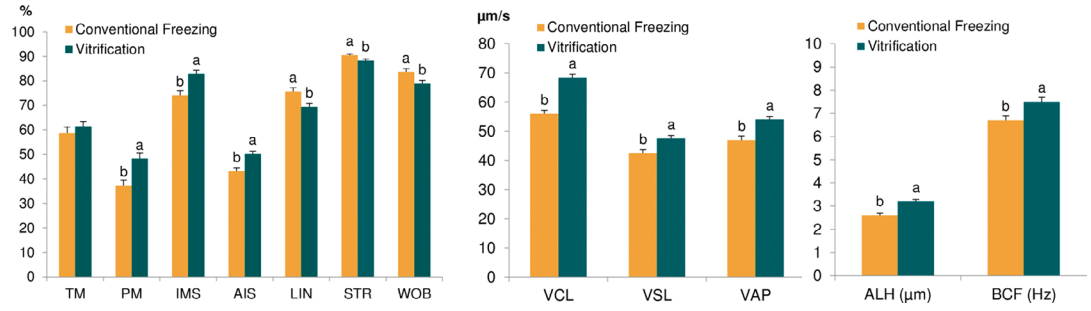

Fig. 1. Comparison of conventional freezing and vitrification in 0.25 ml straws filled with 100 µl at 100 × 10^6 sperm/ml using the extender with 100 mM of trehalose for: TM = total motility, PM = progressive motility, IMS = plasma membrane integrity, AIS = acrosome-intact sperm, LIN = linear coefficient, STR = straightness coefficient, WOB = Wobble coefficient, VCL = curvilinear velocity, VSL = linear velocity, VAP = average path velocity, BCF = beat cross frequency, ALH = lateral head displacement. Different letters (a–b) indicate differences ($P < 0.05$). Values are shown as mean ± SEM.

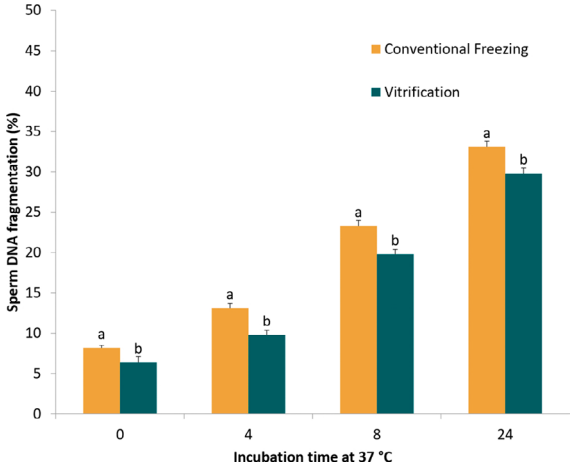

Fig. 2. Comparison of conventional freezing and vitrification on sperm DNA fragmentation (sDF) after 0, 4, 8 and 24 h of incubation; Different letters (a–b) indicate differences ($P < 0.05$). Values are shown as mean ± SEM.

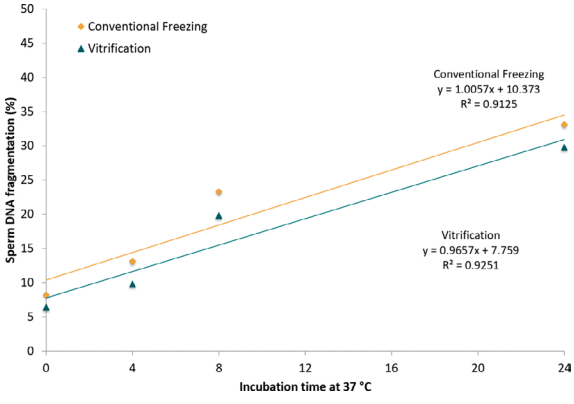

Fig. 3. Effect of vitrification and conventional freezing on the sperm DNA fragmentation rate (sDF%/hour) after 24 h of incubation; no differences were detected in the slopes of sDF between treatment groups ($P > 0.05$).

other species. In this regard, with donkey sperm (Sanchez et al., 2011; Diaz-Jimenez et al., 2017) the optimum molarity is as great as 250 mM of sucrose while with sperm of other species 100 mM molarity is the maximum that can be used, such as those for the Iberian ibex (Pradiee et al., 2015). This is particularly relevant for stallion sperm, which has been previously reported to have a relatively lesser resistance to relatively greater concentrations of carbohydrates (Consuegra et al., 2018a; Hidalgo et al., 2018). In the present study, the molarities of the carbohydrates were selected based on results from previous studies of stallion and human sperm (Schulz et al., 2017; Hidalgo et al., 2018). In the present study, there was the greatest sperm quality with the use of 100 mM of trehalose. This concentration has also been successfully utilized for vitrification of human sperm in straws (Schulz et al., 2017). It should be taken into account, however, that the optimal concentration of carbohydrates differs with use of different vitrification methods, even within the same species. Small volumes of sperm require lesser sugar molarities (20 mM of sucrose) for vitrification when using the spheres method (Hidalgo et al., 2018). This could explain the poor sperm motility obtained in other studies where vitrification in spheres was performed adding molarities above 150 mM (Pérez-Marín et al., 2017; Caturla-Sánchez et al., 2018). Greater carbohydrate concentrations (100 mM) are required for straw vitrification (large volume). This could be attributed to the two main requirements for vitrification: more rapid cooling/warming rates and high viscosity of the solution. When there are larger semen volumes, the cooling/warming processes are slowed down. To compensate for this, a solution of greater viscosity is required for vitrification (Arav et al., 2002), which could be achieved using greater concentrations of carbohydrates (Isachenko et al., 2003).

Furthermore, in the present study a conventional freezing protocol was compared to the vitrification method which resulted in sperm of the greatest quality (straws filled with 100 µl at 100 × 10^6 sperm/ml with the extender containing trehalose 100 mM). With vitrification, there were greater values than with the conventional freezing protocol for most of the sperm variables assessed. These findings are consistent with those from previous studies of human (Aizpurua et al., 2017) and stallion sperm vitrification using the spheres method (Hidalgo et al., 2018). The lesser sperm motility, and integrity of plasma and acrosome membranes with conventional freezing in the present study could be attributed to the osmotic stress placed on the sperm cells during processing. This stress can result from the freezing process (Peña et al., 2011) as well as from the use of an extender that contains permeable cryoprotectants (Ball and Vo, 2001) for which the osmolality is much greater (1162 mOsm/kg) than the extender used for vitrification in the present study (465 mOsm/kg). The greater sDF obtained after thawing in conventional freezing could also be due to this osmotic stress (Yildiz et al., 2010; Kopeika et al., 2015). The hyperosmotic stress could lead to DNA fragmentation as a result of the activation of scavenging enzymes, increasing the formation of free radicals (Grunewald et al., 2009) with this activation being more pronounced when glycerol is included in the extender (Wündrich et al., 2006). Additionally, when samples were exposed to a stressor (incubation at 37 °C for 24 h; (Urbano et al., 2013), there was greater cryptic DNA damage with use of conventional freezing in comparison to vitrification. These different results within the techniques used could be due to the addition BSA to the vitrification extender, a protein usually combined with carbohydrates, which protects DNA by reducing the amount of ROS (Nang et al., 2012).

Vitrification in 0.25 ml straws is a promising alternative to conventional freezing, but for the horse industry it would also be interesting to perform vitrification using larger volumes (0.5 ml straws), which has occurred with human sperm (Isachenko et al., 2011). Unfortunately, many have failed trying to repeat this technology (Katkov et al., 2012) including with stallion sperm, because of the compromised sperm quality after warming (Consuegra et al., 2019; Restrepo et al., 2019). Consequently, further studies are needed to develop this technology.

5. Conclusion

In conclusion, stallion sperm were successfully vitrified in covered 0.25 ml straws filled with a volume of 100 µl of sperm at 100 × 10^6 sperm/ml and by adding 100 mM of trehalose, which resulted in greater sperm quality after warming than with conventional freezing.

Conflicts of interest

None

Acknowledgements

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CHAPTER 1
Stallion sperm freezing with sucrose extenders: A strategy to avoid permeable cryoprotectants
Consuegra et al., 2018. Animal Reproduction Science

CHAPTER 2
Concentrations of non-permeable cryoprotectants and equilibration temperatures are key factors for stallion sperm vitrification success
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CHAPTER 3
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CHAPTER 4

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Consuegra et al., 2020. Animal Reproduction Science

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SUPPLEMENT ARTICLE

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Low-density lipoproteins and milk serum proteins improve the quality of stallion sperm after vitrification in straws

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Abstract

Lipids and proteins can be used for sperm vitrification to preserve the integrity of sperm membranes or to increase the viscosity of the medium. This study evaluated the effect of low-density lipoproteins (LDL) and milk serum proteins (Pronexcell) for stallion sperm vitrification. Hippex extender (Barex Biochemical Products, The Netherlands), plus 1% of bovine serum albumin and 100 mM of trehalose, was used as control for sperm vitrification. In experiment 1, different concentrations of LDL (L1 = 0.25, L2 = 0.5, L3 = 1%) and in experiment 2 of Pronexcell (P1 = 1, P2 = 5, P3 = 10%) were added to control extender. Vitrification was performed in 0.25-ml straws directly plunged into liquid nitrogen. Total motility (TM, %) and progressive motility (PM, %) were analysed by CASA, and plasma membrane (IMS, %) and acrosome membrane integrity (AIS, %) were assessed under epifluorescence microscopy. Post-warmed sperm parameters were compared between treatments by ANOVA. Results were expressed as mean ± SEM. In both experiments, the minimum concentration of LDL and Pronexcell obtained significantly higher values ($p < 0.01$) than the control extender for TM (L1 = 52.95 ± 4.4; P1 = 58.99 ± 4.6; C = 30.88 ± 3.0), PM (L1 = 36.79 ± 5.5; P1 = 47.25 ± 4.3; C = 19.20 ± 2.4), IMS (L1 = 68.88 ± 3.6; P1 = 47.25 ± 4.3; C = 52.81 ± 2.6) and AIS (L1 = 45.88 ± 3.6; P1 = 47.25 ± 4.3; C = 26.00 ± 2.1). No differences in sperm parameters were found among different concentrations of LDL or Pronexcell. In conclusion, the addition of 0.25% LDL and 1% Pronexcell to the vitrification extender is recommended to improve the quality of stallion sperm after vitrification.

KEYWORDS

cryopreservation, horse, LDL, proteins, semen

1 | INTRODUCTION

Kinetic sperm vitrification is performed by direct immersion into liquid nitrogen (LN_2) of low volume semen samples, using carbohydrates and proteins as cryoprotectants (Isachenko, Isachenko, Katkov, Rahimi, et al., 2004). In this sense, stallion sperm has been successfully vitrified following the spheres (Hidalgo et al., 2018) and straws methods (Consuegra et al., 2018) using sucrose and bovine serum albumin (BSA).

Proteins such as human serum albumin (Isachenko, Isachenko, Katkov, Montag, et al., 2004) and BSA (Diaz-Jimenez et al., 2018; Pradiee et al., 2015) are used for sperm vitrification to increase the viscosity of the medium and the glass transition temperature (Isachenko, Isachenko, Katkov, Rahimi, et al., 2004). Serum of cow milk contains BSA as well as β-lactoglobulin, that have also demonstrated a protective effect on stallion sperm during cooling (Batellier, Magistrini, Fauquant, & Palmer, 1997). Pronexcell is a mixture of serum milk proteins, which

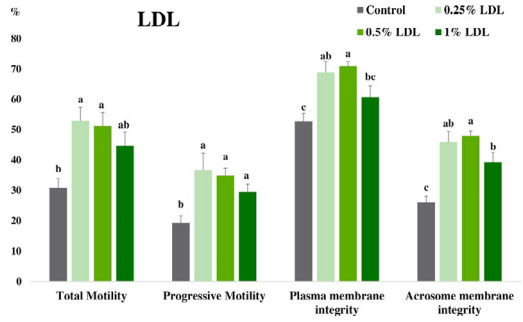

FIGURE 1 Comparison of different concentrations of LDL added to Hippex extender (control) for stallion sperm vitrification. Different letters (a–c) indicate significant differences ($P < 0.01$)

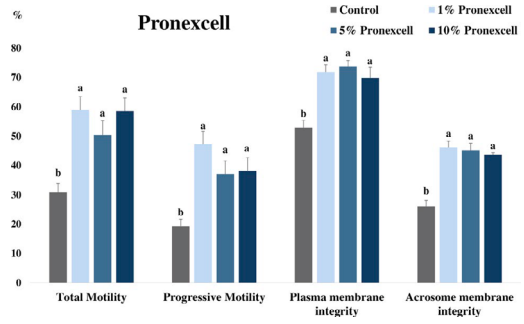

FIGURE 2 Comparison of different concentrations of milk serum proteins added to Hippex extender (control) for stallion sperm vitrification. Different letters (a,b) indicate significant differences ($P < 0.001$)

contains β-lactoglobulin and BSA, and is present in Hippex, a commercial extender for cooling stallion sperm (Crespo et al., 2018). The protective effects of these proteins make them attractive for stallion sperm vitrification. However, the proper concentration of Pronexcell for stallion sperm vitrification remains unknown.

Other additives used for sperm preservation are the low-density lipoproteins (LDL), present in egg yolk plasma (Peruma, 2018). These lipoproteins can replace the phospholipids of the membranes, which are lost during cryopreservation, and preserve intactness of sperm membranes (Peruma, 2018). To the best of our knowledge, LDL have never been added to extenders for sperm vitrification.

Therefore, the aim of this study was to assess different concentrations of LDL and milk serum proteins for stallion sperm vitrification in straws.

2 | MATERIALS AND METHODS

All procedures have been approved by the Ethical Committee on Animal Experimentation of the University of Cordoba, in compliance with the Regional Government of Andalusia (project no. 31/08/2017/105) and the Spanish law for animal welfare and experimentation (RD 53/2013). Hippex extender, LDL and Pronexcell were supplied by Barex Biochemical (Barex Biochemical Products, the Netherlands).

Semen samples ($n = 8$) were collected during the breeding season from four fertile stallions aged from 5 to 23 years, using an artificial vagina. All the ejaculates had physiological mean values for gel-free volume = 43 ml; concentration = 261 × 10^6 sperm/ml; total motility (TM) = 90.7% and progressive motility (PM) = 75.2%; plasma membrane integrity (IMS) = 93%; acrosome membrane integrity (AIS) = 75%. After collection, semen samples were centrifuged (10 min/600 *g*) and the sperm pellets resuspended (100 × 10^6 sperm/ml) in Hippex, adding 1% BSA (w/v) and 100 mM trehalose as control extender for sperm vitrification (C) (Consuegra et al., 2019). In experiment 1, different concentrations of LDL (L1 = 0.25%, L2 = 0.5%, L3 = 1%; w/v) and in experiment 2 of Pronexell (P1 = 1%, P2 = 5%, P3 = 10%; w/v) were added to the control extender, respectively.

Vitrification was performed according to Consuegra et al. (2018). Briefly, 0.25-ml French straws were filled with the different sperm suspensions, inserted into 0.5-ml straws and then plunged into LN_2. For warming, 0.25-ml straws were immersed in 3 ml of extender at 43°C.

Total motility and PM were analysed using the Sperm Class Analyzer (Microptic S.L., Spain). IMS was assessed using Vitaltest kit (Halotech DNA SL, Spain) and AIS using propidium iodide (PI)/peanut agglutinin–fluorescein isothiocyanate (FITC-PNA) stain, both under epifluorescence microscopy. Sperm parameters were compared between treatments by ANOVA followed by Duncan test. Results were expressed as mean ± standard error of the mean. The level of significance was set at $p < 0.05$.

3 | RESULTS

In experiment 1, the lower concentration of LDL (0.25%) obtained higher values ($P < 0.01$) for TM (L1 = 52.95 ± 4.4%), PM (L1 = 36.79 ± 5.5%), IMS= (L1 = 68.88 ± 3.6%) and AIS (L1 = 45.88 ± 3.6%) in comparison with control extender. No differences were found among 0.25 and 0.5% of LDL treatments. For IMS and AIS, significant lower values were obtained with 1% of LDL treatment and control (Figure 1).

In experiment 2, the minimum concentration of Pronexcell (1%) obtained higher values ($p < 0.001$) for TM (P1 = 58.99 ± 4.6%), PM (P1 = 47.25 ± 4.3%), IMS (P1 = 71.88 ± 2.5%) and AIS (P1 = 46.06 ± 2.3%) than control extender. No differences were found among 1, 5 and 10% of Pronexcell (Figure 2).

4 | DISCUSSION

In this study, LDL and Pronexcell (milk serum proteins) increased the values of the sperm parameters obtained after vitrification and warming.

With respect to LDL, all the concentrations assessed improved sperm motility and preserved plasma and acrosome membranes. Previous studies have demonstrated that the phospholipids of LDL create a protective film over the surface of plasma and acrosome membranes (Moussa, Martinet, Trimeche, Tainturier, & Anton, 2002). These phospholipids can also replace the lipids of sperm membranes, which are lost during cryopreservation (Peruma, 2018). It is also known that sperm motility partially depends on membrane transport (Peruma, 2018). Therefore, the protective effect of LDL over sperm membranes can be associated with the preservation of sperm motility observed in this study.

Likewise, the addition of milk serum proteins to the control extender, at any concentration assessed in this study, increased both sperm motility and membrane integrity. A previous study showed that milk proteins improve the post-warmed sperm motility by increasing the activity of GAPDH, key enzyme for glycolysis (Fu et al., 2017). The pathway for ATP production in stallion sperm is not only the oxidative phosphorylation but also glycolysis (Davila et al., 2016). Milk proteins can sequestrate the proteins present in seminal plasma, which extract the lipids from membranes, preserving the integrity of membranes (Plante, Lusignan, Lafleur, & Manjunath, 2015).

Since no differences were found among concentrations of LDL and milk serum proteins in terms of sperm parameters, the lowest concentration of both seems to be adequate to improve the sperm quality after vitrification. Further studies are needed to assess whether or not lesser concentrations of LDL and milk serum proteins improve also stallion sperm vitrification.

In conclusion, the addition of 0.25% LDL or 1% Pronexcell to the vitrification extender is recommended to improve the quality of stallion sperm after vitrification.

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CONFLICT OF INTEREST

None of the authors have any conflict of interest to declare.

AUTHOR CONTRIBUTIONS

M Hidalgo and C Consuegra contributed to all sections. F Crespo and J Dorado contributed to the study design, preparation and revision of the manuscript. M Diaz-Jimenez and B Pereira performed the experiments. All the authors were involved in revision and approval of the final version of the manuscript.

DATA ACCESSIBILITY

Research data are not shared.

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Fertilizing capacity of vitrified stallion sperm assessed utilizing heterologous IVF after different semen warming procedures

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ABSTRACT

The aim of this study was to evaluate the fertilizing capacity of frozen or vitrified stallion sperm after assessing different warming procedures. In Experiment 1, different warming procedures were compared after sperm vitrification: immersion in extender at 43 °C (C), or in a water bath at 37 °C/30 s (W37), 43 °C/10 s (W43) or 60 °C/5 s (W60). With the W60 treatment, there were greater values ($P < 0.05$) for VCL (83.93 ± 3.6 μm/s) and ALH (3.00 ± 0.2 μm) than freezing and with the C group, and greater values ($P < 0.001$) for PM (35.33 ± 2.5 %) than with the W43 treatment. In Experiment 2, the fertilizing capacity of vitrified and frozen sperm was assessed utilizing heterologous IVF procedures, using cattle oocytes. Vitrification resulted in greater values ($P < 0.05$) than freezing for the number of bound sperm (1.36 ± 0.3 and 0.69 ± 0.2, respectively). There were no differences between frozen or vitrified sperm in pronuclear formation (26 hours post-insemination - hpi; 14.08 ± 4.2 % and 22.78 ± 4.8 %, respectively) or cleavage rate (32.77 ± 4.3 % and 39.66 ± 4.6 %, respectively). In conclusion, vitrified stallion sperm warmed in a water bath at 60 ºC had the capacity to penetrate cattle oocytes, leading to pronuclear formation and hybrid embryo cleavage after heterologous IVF.

1. Introduction

Kinetic vitrification of sperm has been a procedure that was developed as an alternative to conventional freezing for cryopreservation of sperm (Isachenko et al., 2011; Aizpurua et al., 2017; Pabón et al., 2019). By conducting this procedure, there is a rapid cooling rate to obtain a glass-like solidification state of sperm cells (Isachenko et al., 2003). This vitrification method was initially performed for human sperm, placing small volumes of semen in liquid nitrogen (LN_2) and using permeable cryoprotectant-free extenders (Isachenko et al., 2008). Sperm vitrification has also been utilized for cryopreservation of stallion sperm (Hidalgo et al., 2018). For stallions sperm, vitrification has been conducted in spheres (Isachenko et al., 2008) and straws (Consuegra et al., 2019a; Consuegra

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et al., 2019b). Vitrification of stallions sperm in straws has been performed either using 0.25 ml (Consuegra et al., 2019b) or 0.5 ml (Consuegra et al., 2019a) straws. Unfortunately, this method has been effective only with cryopreservation in 0.25 ml straws (Consuegra et al., 2019b), because with cryopreservation in 0.5 ml straws the sperm have little motility after warming (Consuegra et al., 2019a).

The warming regimen for the contents in 0.25 ml vitrified straws consists of immersion of the unsealed straws in a commercial milk-based extender at 43 °C (Consuegra et al., 2019b). After imposing this procedure, it is necessary to centrifuge the solution to concentrate the sperm for maintaining viable sperm after thawing. The sperm centrifugation procedure results in an additional cost and is time-consuming. To omit this process, the warming procedure could be performed by immersion of the straw in a water bath because this procedure was previously utilized in a study in which there was thawing of vitrified ram sperm with there being sperm viability after warming (Zilli et al., 2018). It is important to consider that the warming procedure is an important process for effective use of vitrified sperm (Mazur and Seki, 2011), due to the risk of recrystallization of intracellular ice (Seki and Mazur, 2008). In previous studies evaluating sperm vitrification, when there were greater rates of warming (greater temperature and lesser time for thawing) there were greater values for sperm motility and plasma membrane integrity than when the rate of warming was less (Mansilla et al., 2016; Pradiee et al., 2017). These findings may be the reason for the positive results in previous studies when there was thawing of vitrified sperm of different wild ruminants that were stored in spheres when there was warming at temperatures as great as 60 to 65 °C (Pradieé et al., 2018; O'Brien et al., 2019). Furthermore, vitrified stallion sperm stored in 0.5 ml straws have also been warmed in a water bath at 60 °C (Consuegra et al., 2019a). To the best of our knowledge, however, these temperatures have not been evaluated for warming of vitrified stallion sperm stored in 0.25 ml straws.

The spermiogram and *in vitro* fertilization (IVF) represent the most adequate approaches for evaluation of the fertilizing capacity of a sperm sample *in vitro* because these methods include evaluation of gamete interactions, sperm penetration, pronuclear formation and early embryo development (Brahmkshtri et al., 1999). It, however, is well known that homologous IVF evaluations are unsuccessful for use with evaluating stallion sperm (Choi et al., 1994; Hinrichs et al., 2002; Roasa et al., 2007). The small IVF rates are related to the lack of sperm penetration into horse oocytes *in vitro* (Sessions-Bresnahan et al., 2014). These suboptimal IFV rates could be the result of either incomplete sperm capacitation *in vitro* (Tremoleda et al., 2003) or inadequate maturation of oocytes *in vitro* (Hinrichs et al., 2002).

To assess the fertilizing capacity of stallion sperm, the use of the heterologous IVF procedure may be an effective procedure. This method has been successfully performed to assess the capacity of sperm to fertilize oocytes *in vitro* in different species, including red deer, wild goats, sheep and dolphins (Soler et al., 2008; Sánchez-Calabuig et al., 2015; Pradieé et al., 2018; Galarza et al., 2019). There was evaluation of conventionally frozen stallion sperm, utilizing the heterologous IVF procedures using either hamster (Matsukawa et al., 2002), pig (Balao da Silva et al., 2013) or cattle oocytes (Sessions-Bresnahan et al., 2014; de Vasconcelos Franco et al., 2016; Al-Essawe et al., 2018). To the best of our knowledge, the fertilizing capacity of vitrified stallion sperm has not been previously assessed using heterologous oocytes for IVF procedures.

The aim of the present study, therefore, was to optimize the warming regimen for vitrified stallion sperm using different methods and temperatures. After the proper warming regimen was selected, the fertilizing capacity of vitrified, as compared with conventionally frozen, stallion sperm samples was assessed utilizing heterologous IVF procedures.

2. Materials and methods

This study was approved by the Ethical Committee for Animal Experimentation of the University of Cordoba, in compliance with the Regional Government of Andalusia (project no. 31/08/2017/105) and the Spanish law for animal welfare and experimentation (RD 53/2013). All chemicals used were purchased from Sigma Aldrich (Merck, Darmstadt, Germany) unless otherwise stated. The basic medium used for sperm processing was a commercial milk-fraction-based extender (INRA96, IMV Technologies, L'Aigle, France) with trehalose being added at 100 mM (mmol/l) and 1 % of bovine serum albumin (BSA) for sperm vitrification (465 mOsm/kg). For conventional freezing, Gent extender was used (Minitube, Tiefenbach, Germany; 1001 mOsm/kg). Osmolalities of the extenders were assessed using a freezing-point digital micro osmometer Type 6 (Löser Messtechnik, Berlin, Germany). The medium for oocyte maturation was TCM-199 supplemented with 10-ng/ml EGF and 10 % (v:v) fetal calf serum (FCS). The medium for IVF was FERT (FERT-TALP medium [Merck] supplemented with 25 mM bicarbonate, 22 mM sodium lactate, 1 mM sodium pyruvate, 6 mg/ml fatty acid-free BSA and 10 μg/ml heparin).

2.1. Animals

Five stallions of different breeds (three Purebred Spanish Horse, one Arabian and one French warmblood "Selle Français"), aged from 6 to 24 years, clinically healthy and fertile were used as semen donors. Animals were housed in individual paddocks located at the Equine Breeding Centre of the Spanish Army located in Avila, Spain (40.67 °N, 4.69 °W).

2.2. Semen collection and processing

Semen samples were obtained using a Missouri-model artificial vagina in the presence of a mare in estrus. Semen was collected from each animal during the breeding season once or twice per week with there being a total of 15 ejaculates collected for conducting the present study (three ejaculates per animal). All ejaculates collected were evaluated before freezing using procedures subsequently described in this manuscript. Physiological values (mean ± SEM) for gel-free volume (49.3 ± 5.8 ml) were determined in a graduated

collector and sperm concentration (254.9 ± 19.4 × 10^6 sperm/ml) was assessed using a sperm photometer (Spermacue, Minitube), and total (88.9 ± 1.7 %). Progressive sperm motility (71.8 ± 1.7 %) was also assessed as subsequently described in this manuscript. Fresh semen was diluted with the milk based extender at 37 °C (1:1/v:v) and centrifuged for 10 min at 600 x *g*. The sperm pellets were re-suspended to a final concentration of 100 × 10^6 sperm/ml in two different extenders, one for sperm vitrification and other for conventional freezing.

2.3. Vitrification and warming of sperm

Sperm vitrification was conducted in covered 0.25 ml straws according to Consuegra et al. et al., (2019b). Samples extended in the vitrification media were cooled for 1 hour at 5 °C, then loaded in 0.25 ml French straws with a micropipette and horizontally inserted in 0.5 ml straws. Both ends of the external straw were subsequently heat-sealed using a SYMS I sealer (Cryo Bio System, L'Aigle, France). The closed straw was strictly maintained in a horizontal position, and completely immersed into a styrofoam box containing liquid nitrogen (LN_2). After a few seconds, the straw was vitrified. Post-warming analysis was conducted after 24 h of storage in LN_2 containers. For warming, the ends of the external straws were cut, 0.25 ml straws were removed from the 0.5 ml-covering straws and were assigned to four different warming treatments (see experimental design). After warming, the inner straw was immersed in pre-warmed milk-based medium to dilute the sperm suspensions to a final concentration of 25 × 10^6 sperm/ml.

2.4. Conventional sperm freezing and thawing

Semen samples were frozen using a standard regimen for stallions with modifications occurring (Consuegra et al., 2018). Briefly, centrifuged sperm pellets were re-suspended in Gent extender with there being a final concentration of 100 × 10^6 sperm/ml, cooled to 5 °C within 2 hours, loaded in 0.5 ml straws and heat-sealed as previously indicated. The straws were frozen horizontally in racks placed 4 cm above the surface of liquid nitrogen (LN_2) for 10 minutes and stored in LN_2 tanks for at least 24 h.

2.5. Post-warming sperm evaluation

Sperm motility was objectively evaluated using the CASA system (SCA v6.4 Microptic S.L., Spain). Settings were as follows: one photograph every 40 ms, cell size from 15 to 75 $\mu m^{2;}$ connectivity 12; sperm motility was considered progressive when curvilinear velocity was greater than 90 μm/s and straightness greater than 75 %. Two drops of 5 μl each and three microscopic fields per drop were evaluated at random for each sample using a Makler counting chamber (Sefi Medical Instruments Ltd., Haifa, Israel). There were evaluations of a minimum of 200 sperm for the following kinematic variables: total (TM, %) and progressive motility (PM, %), curvilinear (VCL, μm/s), straight line (VSL, μm/s) and average path velocities (VAP, μm/s), amplitude of lateral head displacement (ALH, μm) and beat cross frequency (BCF, Hz).

The integrity of plasma membrane was assessed using the Vital test (Halotech, Madrid, Spain) which is a membrane exclusion assay utilizing a fluorescence microscopy as previously described (Cortés-Gutiérrez et al., 2008). Briefly, a 10 μl aliquot of diluted sperm (25 × 10^6 sperm/ml) were placed on a microscope slide and mixed with 1 μl of acridine orange (AO) and 1 μl of propidium iodide (PI). At least, 200 sperm were evaluated, with spermatozoa staining green being considered to have intact membranes. The percentage of sperm with an intact membrane was recorded (IMS, %).

Acrosome integrity was evaluated using the PI/peanut agglutinin–fluorescein isothiocyanate double stain in an ethanol-fixed sperm smear (Dorado et al., 2014). At least 200 sperm were evaluated on each slide. Sperm with an intact acrosome membrane (AIS, %) had green fluorescence on the anterior region of the sperm head.

Sperm DNA fragmentation was assessed using the sperm chromatin structure assay (SCSA) utilizing the CytoFlex Flow Cytometer (Beckman Coulter, Brea, CA, United States). After thawing/warming, samples of 200 μl and 25 × 10^6 spermatozoa/ml were placed in Eppendorf tubes from each treatment and stored at -80 °C. Chromatin integrity was assessed in each sample as previously described by Love (2014). A total of 5,000 cells were evaluated per sample. The percentage of cells with single-stranded DNA was recorded as the DNA fragmentation index (DFI, %).

2.6. Heterologous in vitro fertilization (Bos taurus oocytes x Equus caballus sperm)

The fertilizing capacity of stallion sperm either frozen or vitrified was evaluated by performing heterologous *in vitro* fertilization (IVF) procedures, using cumulus-oocyte complexes (COCs) from ovaries of cattle collected at a slaughterhouse using previously described procedures (Pradieé et al., 2018). Oocyte *in vitro* maturation was performed in 60 μl drops (30 COCs per drop) of maturation medium for 24 h at 38.5 °C when there was an atmosphere of 5 % CO_2 in air at maximum humidity.

After 24 h, matured oocytes were washed twice in FERT medium and transferred to 30 μl drops of FERT (30 COCs per drop). For heterologous IVF, in each group (conventional freezing and vitrification), one single ejaculate from each of four stallions was used. Semen samples were pooled and the experiment was replicated six times in each group (technical replicates). The sperm quality of frozen and vitrified pooled semen was evaluated after warming. In addition, frozen-thawed sperm from a single Asturian Valley bull (Asturgen, Gijon, Spain) with proven fertility was used as control in each replicate as previously described (Sánchez-Calabuig et al., 2015). Colloid single-layer centrifugation, using Equipure for stallion sperm or Bovipure for bull sperm (Nidacon International, Gothenburg, Sweden), was conducted to enrich the population of motile and live sperm from each group prior to heterologous IVF. Sperm were diluted in FERT medium, and 30 μl of this sperm suspension was added to each fertilization drop with there being a final

concentration of 1×10^6 sperm/ml.

In each IVF replicate, four groups were assessed: two heterologous groups of stallion sperm, frozen-thawed (He frozen) and vitrified-warmed (He vitrified), a homologous control group using bull sperm (Ho), and a parthenogenetic control group (containing only matured COCs). A total of 1,410 cumulus-oocyte complexes (COCs) were divided in these four groups: Ho ($n = 264$), He frozen ($n = 534$), He vitrified ($n = 541$) and parthenogenetic control ($n = 71$) groups. Every IVF variable was assessed on a subset of oocytes of each group. Number of oocytes used for evaluation of each variable and group is provided in brackets in Table 3.

Sperm and matured oocytes were co-incubated at 38.5 °C when there was an atmosphere of 5 % CO_2 in air with maximum humidity. Sperm-oocyte interactions were assessed using the sperm-zona pellucida binding assay at 2.5 h post-insemination (hpi). For this assay, oocytes were vortexed for 3 min. Oocytes were subsequently fixed and stained with Hoechst 33342 to determine the number of sperm that remained bound to the zona pellucida using a Nikon Eclipse E200 epifluorescence microscope (UV-2E/C excitation: 340-380 nm, emission: 435-485 nm). The IVF capacity of sperm was evaluated by observing pronuclei formation at 18 h for homologous samples as the control to ensure that the IVF was properly performed, considering 18 h is a long enough period of time to observe pronuclear formation in this group. For heterologous IVF, evaluations for pronuclei formation were made at 18, 20, 22, 24 and 26 hpi for heterologous IVF. For this evaluation, presumptive zygotes were treated and examined using procedures previously described in this manuscript for sperm-zona binding. The cleavage rate was evaluated at 48 hpi in all groups.

2.7. Experimental design

2.7.1. Experiment 1. Effect of different warming methods for vitrification of stallion sperm using straws in comparison to conventional freezing

After semen processing, sperm ($n = 15$) were placed in the vitrification or freezing medium at a concentration of 100×10^6 sperm/ml. Conventional freezing and sperm vitrification were performed using procedures previously described in this manuscript. Conventionally frozen straws were thawed by immersion in a water bath at 37 °C for 30 s and resuspended with the milk-based extender. For warming of vitrified samples, 0.25 ml straws were removed from the 0.5 ml-covering straws and were assigned to be evaluated after four different warming procedures were imposed. The first procedure was used for the control sample which consisted of the immersion of 0.25 ml straws in a tube containing a commercial milk-based extender at 43 °C. The unsealed straws were immersed in a vertical position with the cotton edge at the top. The sperm suspensions were subsequently centrifuged at 600 x *g* for 10 min (C) (Consuegra et al., 2019b). The other warming methods were conducted by immersion of the 0.25 ml straw in a water bath at 37 °C for 30 seconds (W37) (Jiménez-Rabadán et al., 2015; Diaz-Jimenez et al., 2019) at 43 °C for 10 seconds (W43) (Schulz et al., 2017) or 60 °C for 5 seconds (W60) (Pradiee et al., 2015). With this method, it is important to keep the 0.25 ml straw in vertical position, placing the end of the straw containing the cotton into the water and the unsealed end of the straw out of the water using dissection forceps. Post-warming, sperm variables (TM, PM, IMS, AIS, VCL, VSL, VAP, ALH and BCF) were assessed and compared when there was evaluation of conventional freezing and vitrification using different warming procedures. The warming regimen for which there were the most promising results was used in Experiment 2 for comparison of sperm viability variables after there was warming of vitrified stallion sperm with comparisons being made to that of conventionally cryopreserved stallion sperm.

2.7.2. Experiment 2. Evaluation of the fertilizing capacity of vitrified or conventionally frozen sperm utilizing heterologous IVF

The warming treatment resulting in the greatest sperm characteristics of vitrified sperm (W60 = 60 °C/5 s) was and compared with warming of semen that had been frozen using conventional procedures. The sperm variables (TM, PM, IMS, AIS, VCL, VSL, VAP, ALH, BCF and DFI) of stallion pooled sperm (frozen-thawed and vitrified-warmed) were evaluated after warming and before colloid single-layer centrifugation and IVF. To assess the fertilizing capacity of vitrified and frozen stallion sperm, heterologous IVF was performed with cattle oocytes as previously described. Sperm zona binding, pronuclei formation and cleavage rate were recorded and compared

Table 1
Sperm variables assessed in stallion sperm vitrified in straws after warming using different regimens in comparison to conventional freezing; Three ejaculates were assessed from each of five stallions ($n = 15$)

Variables	Conventional Freezing	Vitrification				*P*-value
		C	W37	W43	W60	
	Water bath 37 °C/30 s	Extender 43 °C	Water bath 37 °C/30 s	Water bath 43 °C/10 s	Water bath 60 °C/5 s	
TM (%)	49.75 ± 2.8^{a}	51.20 ± 2.7^{a}	41.27 ± 4.9^{b}	45.36 ± 3.2^{ab}	52.93 ± 3.3^{a}	<0.05
PM (%)	35.50 ± 2.2^{a}	38.60 ± 2.0^{a}	21.80 ± 2.7^{c}	29.02 ± 2.6^{b}	35.33 ± 2.5^{a}	<0.001
IMS (%)	66.00 ± 1.4^{a}	68.53 ± 2.2^{a}	58.07 ± 2.8^{b}	65.37 ± 1.9^{a}	71.33 ± 2.9^{a}	<0.01
AIS (%)	41.81 ± 1.8	47.23 ± 1.8	42.47 ± 1.7	44.27 ± 2.2	47.47 ± 1.1	>0.05
VCL (μm/s)	71.81 ± 2.4^{b}	71.53 ± 2.0^{b}	73.00 ± 3.7^{b}	82.22 ± 3.2^{a}	83.93 ± 3.6^{a}	<0.01
VSL (μm/s)	53.81 ± 1.8^{a}	54.53 ± 1.3^{a}	42.73 ± 3.0^{b}	49.71 ± 2.5^{a}	51.73 ± 2.3^{a}	<0.01
VAP (μm/s)	63.75 ± 2.3^{a}	62.73 ± 1.8^{a}	52.60 ± 3.6^{b}	60.43 ± 2.9^{a}	63.33 ± 2.9^{a}	<0.01
ALH (μm)	2.38 ± 0.1^{b}	2.33 ± 0.1^{b}	2.47 ± 0.1^{ab}	2.92 ± 0.2^{a}	3.00 ± 0.2^{a}	<0.05
BCF (Hz)	7.25 ± 0.3^{ab}	6.80 ± 0.2^{bc}	6.60 ± 0.3^{c}	7.40 ± 0.3^{ab}	7.53 ± 0.3^{a}	<0.05

TM = total motility; PM = progressive motility; IMS = plasma membrane integrity; AIS = acrosome-intact sperm; VCL = curvilinear velocity; VSL = linear velocity; VAP = average path velocity; ALH = lateral head displacement; BCF = beat cross frequency; Different superscripts (a, b, c) indicate differences in values among warming procedures ($P < 0.05$); Results are expressed as mean ± SEM

among treatment groups.

2.8. Statistical analysis

For each variable, normality and homogeneity of variances were assessed using the Kolmogorov-Smirnov and Levene test, respectively. When values were not normally distributed, results were transformed to a logarithmic scale. Results were expressed as mean ± standard error of the mean (SEM). There were considered to be mean differences when there was a $P < 0.05$.

Analysis of the sperm data was conducted using SAS v9.0. A general linear model (PROC GLM) followed by Duncan method were performed with animals and ejaculates as random factors. For heterologous IVF, the percentage of bound sperm, male pronucleus formation and cleavage rate were analysed using an ANOVA followed by use of the Tukey's *post hoc* multiple comparison tests. All calculations were made using Statistica for Windows v.12.0 software (StatSoft, Tusla, OK, USA).

3. Results

3.1. Experiment 1. Effect of different warming methods for vitrified stallion sperm using straws in comparison to conventional freezing

The values for sperm variables after warming using different methods for stallion sperm vitrification in straws are provided in Table 1. The VCL and ALH were greater ($P < 0.05$) with the W60 (83.93 ± 3.6 μm/s; 3.00 ± 0.2 μm) warming regimen in comparison to using the regimen for thawing of conventional frozen stallion sperm (71.81 ± 2.4 μm/s; 2.38 ± 0.1 μm and 71.53 ± 2.0 μm/s; 2.33 ± 0.1 μm for the W60 treatment and C, respectively). With the W60 treatment, there was also a greater ($P < 0.001$) PM (35.33 ± 2.5 %) when compared with W43 treatment (29.02 ± 2.6 %). There were the least values for most of the variables that were assessed (except ALH) when there was the W37 treatment for warming of semen samples ($P < 0.05$). There were no differences among treatments for the AIS variable.

3.2. Experiment 2. Evaluation of the fertilizing capacity of vitrified or conventionally frozen sperm using heterologous IVF

The values of sperm characteristics before IVF for stallion pooled sperm (frozen-thawed and vitrified-warmed, technical replicates) are reported in Table 2. Bull sperm had the following mean values for sperm variables after thawing: TM = 77.53 %, PM = 52.05 %, IMS = 61.98 % and AIS = 90.99 %.

Vitrified, as compared with conventionally frozen stallion sperm, had markedly greater respective values for TM (63.54 ± 2.0 and 48.49 ± 4.0, %), PM (50.38 ± 1.8 and 35.56 ± 3.0, %), VCL (87.67 ± 1.7 and 73.83 ± 2.2, μm/s), VSL (61.72 ± 1.3 and 54.31 ± 0.6, μm/s), VAP (70.22 ± 1.6 and 60.25 ± 1.4 μm/s) and ALH (2.61 ± 0.1 and 2.18 ± 0.1, μm/s). There were no differences in respective values for vitrified, compared with conventionally frozen sperm for IMS (84.92 ± 1.1 and 81.58 ± 1.7, %), AIS (69.42 ± 3.8 and 68.68 ± 2.3, %), BCF (9.01 ± 0.2 and 8.71 ± 0.2, Hz) and DFI (24.21 ± 5.6 and 21.53 ± 2.8, %).,

Values for results of heterologous IVF are depicted in Fig. 1 and are provided in Table 3. The number of bound sperm was greater for vitrified stallion sperm samples in comparison to conventionally frozen-thawed stallion sperm and to the oocytes of the homologous group ($P < 0.05$).

The embryos of the homologous group (Ho) had greater ($P < 0.05$) pronuclei formation at 18 h (63.22 ± 5.2 %) and cleavage rate at 48 h after IVF (75.83 ± 3.9 %) than embryos of the heterologous groups. The values for the embryos of the He frozen group at 18 hpi for pronuclei presence was 13.11 ± 4.4 % and cleavage rate was 32.77 ± 4.3 %. For the embryos of the He vitrified group, the values for pronuclei that were detected at 18 hpi was 13.58 ± 3.8 %; and for cleavage rate was 39.66 ± 4.6 %. Nevertheless, there were no differences in pronuclei formation (18, 20, 22, 24 or 26 h) or cleavage rate among heterologous groups. There was a spontaneous parthenogenetic activation rate of 5.6 ± 2.8 % in mature unfertilized oocytes of cattle at 48 hpi.

Table 2
Quality of pooled sperm from four stallions before IVF for treatments: frozen and vitrified sperm; One single ejaculate from each stallion was pooled; Sperm pooling was replicated six times for each treatment group ($n = 6$).

Variables	Conventional Freezing	Vitrification	*P*-value
TM (%)	48.49 ± 4.0^{b}	63.54 ± 2.0^{a}	<0.01
PM (%)	35.56 ± 3.0^{b}	50.38 ± 1.8^{a}	<0.01
IMS (%)	81.58 ± 1.7	84.92 ± 1.1	>0.05
AIS (%)	68.68 ± 2.3	69.42 ± 3.8	>0.05
VCL (μm/s)	73.83 ± 2.2^{b}	87.67 ± 1.7^{a}	<0.001
VSL (μm/s)	54.31 ± 0.6^{b}	61.72 ± 1.3^{a}	<0.001
VAP (μm/s)	60.25 ± 1.4^{b}	70.22 ± 1.6^{a}	<0.01
ALH (μm)	2.18 ± 0.1^{b}	2.61 ± 0.1^{a}	<0.01
BCF (Hz)	8.71 ± 0.2	9.01 ± 0.2	>0.05
DFI (%)	21.53 ± 2.8	24.21 ± 5.6	>0.05

TM = total motility; PM = progressive motility; IMS = plasma membrane integrity; AIS = acrosome-intact sperm; VCL = curvilinear velocity; VSL = linear velocity; VAP = average path velocity; ALH = lateral head displacement; BCF = beat cross frequency; DFI = DNA fragmentation index; Different superscripts (a, b) indicate differences between treatments ($P < 0.05$); Results are expressed as mean ± SEM

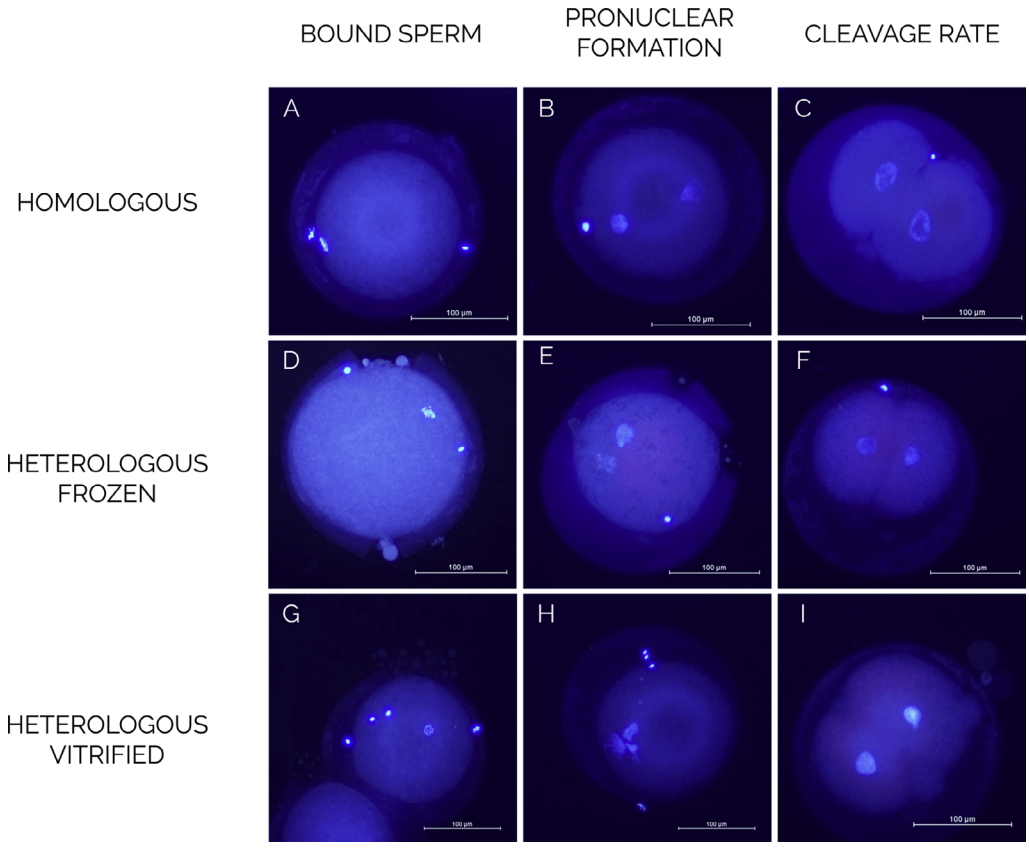

Fig. 1. Evaluation of sperm-oocyte binding, pronuclear formation and cleavage after homologous (bull sperm) and heterologous (frozen-thawed and vitrified-warmed stallion sperm) IVF; Gametes were stained with Hoechst 33342 and visualized using a phase-contrast microscope (x 40 magnification); First line (Homologous): Bound (a) bull sperm after 2.5 hours of coincubation with zona-intact cattle oocytes, pronuclei formation (b) after 18 hpi, and embryo cleavage ***(c)*** after 48 hpi; Second line (Heterologous frozen): Bound (d) frozen stallion sperm, after 2.5 hours of gametes coincubation, pronuclei formation (e) after 24 hpi, and hybrid-embryo cleavage (f) after 48 hpi; Third line (Heterologous vitrified): Bound (g) vitrified stallion sperm, after 2.5 hours of gamete coincubations, pronuclei formation (h) after 24 hpi, and hybrid-embryo cleavage (i) after 48 hpi.

Table 3
Rates of pronucleus formation and cleavage after homologous (bull sperm) and heterologous (frozen-thawed and vitrified-warmed stallion sperm) co-incubation with cattle oocytes at different hour post-insemination (h); Numbers in brackets (*n*) refer to total number of oocytes used in each treatment; The experiment was replicated six times ($n = 1{,}410$ total number of oocytes or presumptive zygotes examined).

Semen groups	Bound sperm	Pronuclear formation (%)					Cleavage rate (%)
	2.5 h	18 h	20 h	22 h	24 h	26 h	
Ho ($n = 264$)	0.53 ± 0.1^{b} ($n = 57$)	63.22 ± 5.2^{a} ($n = 87$)					75.83 ± 3.9^{a} ($n = 120$)
He frozen ($n = 534$)	0.69 ± 0.2^{b} ($n = 55$)	13.11 ± 4.4^{b} ($n = 61$)	15.79 ± 4.2 ($n = 76$)	19.72 ± 4.8 ($n = 71$)	11.11 ± 3.7 ($n = 81$)	14.08 ± 4.2 ($n = 71$)	32.77 ± 4.3^{b} ($n = 119$)
He vitrified ($n = 541$)	1.36 ± 0.3^{a} ($n = 59$)	13.58 ± 3.8^{b} ($n = 81$)	13.43 ± 4.2 ($n = 67$)	12.68 ± 4.0 ($n = 71$)	10.29 ± 3.7 ($n = 68$)	22.78 ± 4.8 ($n = 79$)	39.66 ± 4.6^{b} ($n = 116$)
Parthenogenic ($n = 71$)							5.63 ± 2.8^{c} ($n = 71$)

Ho = homologous *in vitro* fertilization with bull sperm; He frozen = heterologous *in vitro* fertilization with frozen stallion sperm; He vitrified = heterologous *in vitro* fertilization with vitrified stallion sperm; Different superscripts (a, b, c) in the same column indicate differences $P < 0.05$; Values are expressed as mean ± SEM.

4. Discussion

This is the first report in which the fertilizing capacity of vitrified stallion sperm has been assessed using heterologous IVF procedures. Interestingly, vitrified stallion sperm had the capacity to penetrate zona intact oocytes of cattle, leading to pronuclear formation and hybrid embryo cleavage.

Prior to assessment of the fertilizing capacity of vitrified sperm, the most effective method for warming the straws containing stallion semen was evaluated. The warming method generally used for vitrified stallion sperm consists of the immersion of the straw in a pre-warmed extender at 43 °C, followed by centrifugation (Consuegra et al., 2019b). Whereas, in the present study the effectiveness of maintaining viability of stallion semen was determined when warming the contents of straws containing the semen in a water bath. This method consists of removing the 0.25 ml straw from the covering straw before it is immersed in the water bath. Consequently, the 0.25 ml straw is in direct contact with the warm water. In addition, to determine the most effective temperatures and times for warming stallion semen and maintaining sperm viability, there was assessment of temperature and time combinations evaluated in previous studies: 37 °C/30 s (Jiménez-Rabadán et al., 2015), 43 °C/10 s (Hidalgo et al., 2018; Consuegra et al., 2019b), 60 °C/5 s (Consuegra et al., 2019a).

Among warming methods, there were the least values when there was warming at 37 °C for 30 s in a water bath. These findings are consistent with those in previous studies with donkey (Diaz-Jimenez et al., 2019) and human (Sanchez et al., 2013; Mansilla et al., 2016) semen. In these studies, for the vitrified sperm samples warmed at 37 to 38 °C there were the least values in comparison to the other cryopreserved semen sample warming regimens using greater temperatures. This warming regimen (37 °C/30 s) is currently used for thawing stallion sperm conventionally frozen in 0.5 ml straws (Oldenhof et al., 2013; Consuegra et al., 2018). When there is vitrification and freezing procedures imposed using different methodologies (volume, packaging, cooling rate, etc.), there is need for different warming procedures. Based on results from the present study, it is apparent that regimen of warming at 37 °C/30 s is not effective for maintaining the viability of vitrified samples after warming of vitrified stallion semen, probably due to the slow warming rate that occurs.

To attain a more rapid warming of vitrified stallion semen samples, greater than typically imposed temperatures for warming of the sample (43 °C and 60 °C) were imposed. Warming at 60 °C tended to result in greater values for plasma membrane integrity and for most of the sperm motility variables assessed when there was imposing of the other warming regimens. This finding is consistent with results from other studies where there was vitrification and warming of semen assessments of sperm viability after imposing of these procedures. In these previous studies, when there was the greatest temperature imposed, there was the greatest values for plasma membrane integrity (Diaz-Jimenez et al., 2019), and also sperm motility (Mansilla et al., 2016; Pradiee et al., 2017). In these previous studies where the human semen was stored in 0.25 ml straws, there was warming at 42 °C (Mansilla et al., 2016), while for other animal species temperatures such as 60 °C (Pradiee et al., 2017) and 70 °C (Diaz-Jimenez et al., 2017) were imposed on spheres and 0.5 ml straws that contained the semen, respectively. Hence, it appears as though the proper warming method depends on the species and package used for vitrification (Diaz-Jimenez et al., 2019). In the case of stallion sperm, when vitrified 0.5 ml straws were warmed using different thawing regimens (42 °C/20 s and 60 °C/15 s), there were no differences in sperm variables subsequent to thawing (Consuegra et al., 2019a). It is important to consider that as volume increases (0.5 ml) a greater temperature is needed for a warming rate that avoids particle recrystallization in sperm cells (Mazur and Seki, 2011). There, was therefore no improvement in sperm viability variables when there was warming of vitrified 0.5 ml straws of stallion sperm at 60 °C, but when this temperature was imposed for warming of 0.25 ml straws, the sperm characteristics were relatively greater than with other procedures.

The warming regimen chosen for Experiment 2 in the present study was the immersion of the cryopreserved stallions semen in a water bath at 60 °C for 5 s for two reasons. Firstly, the method of warming using a water bath is financially economical and there is less labor needed for conducting the procedure because of the time-efficiency for conducting this procedure with there with there being no need for centrifugation of the samples. Secondly, when there was warming of vitrified stallion semen samples at 60 °C in a water bath, there were greater values for sperm variables related to cell viability than when other warming regimens were imposed using a water bath.

The sperm characteristics of the sperm pooled for conducting vitrification and freezing procedures (technical replicates) were evaluated before imposing the IVF procedures. Results from these evaluations of vitrified samples indicated there were greater values for nearly all sperm variables than those for frozen sperm samples. These results from the present study are consistent with those from previous studies where there was stallion sperm vitrification (Hidalgo et al., 2018; Consuegra et al., 2019b). Furthermore, when there was utilization of vitrification procedures, there was the largest number of sperm bound to cattle oocytes in comparison with conventional freezing. There were also similar values when there were evaluations when conducting homologous IVF procedures. It has been suggested that the interaction of gametes does not only depend on there being complementary proteins on sperm and oocytes for binding of sperm ligands to occur, but also other factors such as the capacity of sperm to penetrate the oocyte (related to sperm motility) (Bedford, 2014). Interestingly, ALH and VCL values were greater in vitrified stallion sperm in comparison to sperm samples conventionally frozen. These variables have been associated with sperm hyperactivation and are closely correlated with IVF outcomes (Verstegen et al., 2002). Likewise, sperm binding to oocytes is related with *in vivo* fertility of stallions (Fazeli et al., 1995; Pantke et al., 1995; Meyers et al., 1996) and in the present study there were greater values of bound sperm in the vitrification group.

Fertilizing capacity of vitrified and frozen stallion sperm was also assessed by evaluating pronuclei formation and hybrid embryo cleavage after heterologous IVF. The values for parthenogenetic activation in the present study were similar to those previously reported (Downs, 1990; Sánchez-Calabuig et al., 2015). There, however, were no differences between vitrified or frozen stallion sperm regarding heterologous IVF outcomes. In the present study, frozen and vitrified sperm penetrated cattle oocytes, leading to pronuclear formation and hybrid embryo cleavage after heterologous IVF. Findings from the present study are consistent with those in previous

studies where the fertilizing capacity of frozen stallion sperm was assessed using intact-zona cattle oocytes (Sessions-Bresnahan et al., 2014; de Vasconcelos Franco et al., 2016). In these studies, there was pronuclear formation of 14 % to 26 % after 22 to 26 hpi. Furthermore, 30 % of the cell cleavages occurred after 56 hpi (Sessions-Bresnahan et al., 2014). It is remarkable that in these previous studies there was no pronuclear formation before 22 hpi (Sessions-Bresnahan et al., 2014). This longer than expected time to pronuclear formation was attributed to differences in sperm binding and penetration rates between horses and cattle (Sessions-Bresnahan et al., 2014; Sánchez-Calabuig et al., 2015). Unexpectedly, in the present study the pronuclear formation when there was evaluation of stallion sperm initially occurred only at 18 hpi.

Although with utilization of IVF procedures in the present study, there was pronucleus formation when both frozen and vitrified stallion sperm were evaluated, the greatest percentage of pronuclear formation occurred earlier when there were comparisons of the frozen with the vitrification semen samples. The greatest rate of pronuclear formation was observed at 22 h (19.72 %) when there was evaluation with the frozen semen, while with the vitrified semen there was pronuclear formation at 26 h (22.78 %). This difference in time required for pronuclear formation could be attributed to a longer time required for sperm to transit through the cumulus cells, then bind and penetrate the zona pellucida and oolema (Sessions-Bresnahan et al., 2014). In the present study, however, this does not appear to have contributed to a longer period for pronuclear formation from time of initiation of the IVF procedures because pronuclear formation was observed after only 18 hpi in both groups: frozen 13.1 % and vitrified 13.6 %. The other explanation for this longer period to pronuclear formation after initiation of IVF procedures could be the presence of a more stable chromatin in the vitrified sperm samples. A greater chromatin stability implies there is a greater protection of DNA integrity (Lewis and Aitken, 2005), but also that there is a longer period for DNA decondensation to occur, and thus, formation of the male pronucleus (Perreault et al., 1987; Córdova et al., 2002; Madrid-Bury et al., 2005). In the present study, there were no differences regarding DNA integrity between vitrified or frozen sperm. The longer period to pronuclear formation after initiation of IVF procedures in the present study, therefore, cannot be attributed to a greater DNA fragmentation. The chromatin stability of these sperm, however, has not been evaluated. Other analyses should be performed to assess the likelihood that chromatin to decondensation would occur, such as evaluations of propidium iodide uptake (Molina et al., 1995; Stoll et al., 2013). Further studies are needed to evaluate the DNA stability of vitrified and frozen sperm. Furthermore, after the determination that vitrified stallion sperm has fertilization capacity *in vitro,* it would be interesting to perform further studies using vitrified stallion sperm for artificial insemination of mares.

5. Conclusion

In conclusion, the warming regimen selected for vitrified stallion sperm in Experiment 2 of the present study was the immersion in a water bath at 60 °C for 5 s. Vitrified and conventionally frozen stallion sperm had a similar fertilizing capacity, being able to penetrate cattle oocytes, leading to pronuclear formation and hybrid embryo cleavage after imposing heterologous IVF procedures.

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Author Contributions

Designed the experiment: M Hidalgo. Obtained the individuals to analyse: F Crespo. Collected the samples: C Consuegra and F Crespo. Performed the laboratory analysis: C Consuegra, M Diaz-Jimenez, B Pereira, MJ Sánchez-Calabuig, P Beltrán-Breña, S Pérez-Cerezales. Analysed the data: C Consuegra, M Hidalgo, MJ Sánchez-Calabuig and D Rizos. Wrote the manuscript: C Consuegra and M Hidalgo. Corrected the manuscript: M Hidalgo, J Dorado, D Rizos and F Crespo. Head of the project: M Hidalgo and J Dorado.

Declaration of Competing Interest

The authors declare no conflicts of interest.

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CONCLUSIONES

CONCLUSIONES

Conforme a los objetivos planteados y los resultados obtenidos en la presente Tesis Doctoral se pueden extraer las siguientes conclusiones:

1. El esperma de caballo puede ser congelado en ausencia de crioprotectores permeables, usando una combinación de 100 mM de sacarosa y 1% de BSA como crioprotectores no permeables.

2. El esperma de caballo puede ser vitrificado en pequeños volúmenes (esferas) después del periodo de equilibrado a 5ºC en un diluyente en el que se combina 20 mM de sacarosa y 1% de BSA, obteniendo valores superiores en los parámetros espermáticos que la congelación convencional con glicerol.

3. La vitrificación de esperma de caballo en pajuelas de 0,5 mL obtuvo peor calidad en comparación con la congelación convencional y la vitrificación en esferas. Si bien distintos métodos de calentamiento no mejoraron la calidad del esperma, el uso de la centrifugación coloidal sí puede ser una estrategia para mejorar dicha calidad, ya que se obtuvieron valores más altos de movimiento espermático e integridad de la membrana plasmática. No obstante, la vitrificación en pajuelas de 0,5 mL no puede ser considerada una alternativa a las técnicas convencionales de criopreservación.

4. El esperma de caballo puede ser vitrificado en pajuelas de 0,25 mL, con 100 µl de esperma a una concentración de 100 x 10^6 espermatozoides/mL y empleando un medio con 100 mM de trehalosa y 1% BSA. Igualmente, puede incorporarse al medio 0,25% de LDL o 1% de Pronexcell, a fin de mejorar los parámetros espermáticos. La vitrificación en pajuelas de 0,25 mL resultó en una mejor calidad del esperma en comparación con la congelación convencional.

5. Las dosis vitrificadas de esperma de caballo pueden calentarse en un baño de agua a 60ºC durante 5s como alternativa al método convencional de calentamiento de pajuelas vitrificadas. Las dosis de esperma de caballo vitrificado y congelado convencionalmente mostraron una capacidad fecundante similar, logrando penetrar a los ovocitos bovinos dando lugar a la formación de pronúcleos y a la segmentación de embriones híbridos tras la FIV heteróloga.

CONCLUSIONS

CONCLUSIONS

According to the objectives and the results obtained in the present Doctoral Thesis, we can hereby conclude:

1. Stallion sperm can be frozen in the absence of permeable cryoprotectants, using a combination of sucrose 100 mM and 1% of BSA as non-permeable cryoprotectants agents.

2. Stallion sperm can be vitrified in small volumes (spheres) after equilibration at 5ºC using a combination of 20 mM sucrose and 1% BSA based extender obtaining higher values of sperm parameters than conventional freezing with glycerol.

3.Stallion sperm vitrification in 0.5 mL straws obtained worse sperm quality than conventional freezing and vitrification in spheres. Although different warming procedures did not enhance the sperm quality, SLC may be a strategy to improve the sperm quality since higher values of sperm motility and plasma membrane integrity were obtained with this technique. However, vitrification in 0.5 mL straws cannot be considered an alternative to other conventional cryopreservation techniques.

4. Stallion sperm can be vitrified in 0.25 mL straws, using 100 µl of sperm at a concentration of 100 x 10^6 sperm/mL and using an extender with 100 mM of trehalose and 1% BSA. Likewise, 0.25% LDL or 1% Pronexcell can be added into the extender in order to improve the sperm parameters. Vitrification in 0.25 mL straws resulted in greater sperm quality compared to conventional freezing.

5. Vitrified doses of stallion sperm can be warmed in a water bath at 60ºC for 5s, as an alternative to the conventional warming method for vitrified straws. Vitrified and conventionally frozen stallion sperm had a similar fertilizing capacity, being able to penetrate cattle oocytes, leading to pronuclear formation and hybrid embryo cleavage after imposing heterologous IVF procedures.

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ÍNDICES DE CALIDAD

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PRIMERA PUBLICACIÓN:

Título: Stallion sperm freezing with sucrose extenders: A strategy to avoid permeable cryoprotectants.

Autores (p.o. de firma): C Consuegra, F Crespo, M Bottrel, I Ortiz, J Dorado, M Diaz-Jimenez, B Pereira, M Hidalgo.

Revista (año, vol., pág.): Animal Reproduction Science, 2018, 191, 85-91.

Base de Datos Internacional o Nacional en las que está indexada: Journal of Citation Reports (JCR). 2018.

Área temática en la Base de Datos de referencia: Agriculture, Dairy and Animal Sciences.

Índice impacto año: 1,817.

Posición de la revista / n.º de revistas en el área temática: 12/61(Q1).

SEGUNDA PUBLICACIÓN:

Título: Concentrations of non-permeable cryoprotectants and equilibration temperatures are key factors for stallion sperm vitrification success.

Autores (p.o. de firma): M Hidalgo, C Consuegra, J Dorado, M Diaz-Jimenez, I Ortiz, B Pereira, R Sanchez, F Crespo.

Revista (año, vol., pág.): Animal Reproduction Science, 2018, 196, 91-98.

Base de Datos Internacional o Nacional en las que está indexada: Journal of Citation Reports (JCR). 2018.

Área temática en la Base de Datos de referencia: Agriculture, Dairy and Animal Sciences.

Índice impacto año: 1,817.

Posición de la revista / n.º de revistas en el área temática: 12/61(Q1).

TERCERA PUBLICACIÓN:

Título: Vitrification of large volumes of stallion sperm in comparison to spheres and conventional freezing: effect of warming procedures and sperm selection.

Autores (p.o. de firma): C Consuegra, F Crespo, J Dorado, M Diaz-Jimenez, B Pereira, I Ortiz, R Arenas, J Morrell., M Hidalgo.

Revista (año, vol., pág.): Journal of Equine Veterinary Science, 2019, 83.102680.

Base de Datos Internacional o Nacional en las que está indexada: Journal of Citation Reports (JCR). 2018.

Área temática en la Base de Datos de referencia: Veterinary Sciences.

Índice impacto año: 1,100.

Posición de la revista / n.º de revistas en el área temática: 75/141(Q3).

CUARTA PUBLICACIÓN:

Título: Comparison of different sucrose based extenders for stallion sperm vitrification in straws.

Autores (p.o. de firma): C Consuegra, F Crespo, J Dorado, I Ortiz, M Diaz-Jimenez, B Pereira, M Hidalgo.

Revista (año, vol., pág.): Reproduction in Domestic Animals, 2018, 53, 57-59.

Base de Datos Internacional o Nacional en las que está indexada: Journal of Citation Reports (JCR). 2018.

Área temática en la Base de Datos de referencia: Veterinary Sciences.

Índice impacto año: 1,638.

Posición de la revista / n.º de revistas en el área temática: 36/141(Q2).

QUINTA PUBLICACIÓN:

Título: Vitrification of stallion sperm using 0.25 mL straws: Effect of volume, concentration and carbohydrates (sucrose/trehalose/raffinose).

Autores (p.o. de firma): C Consuegra, F Crespo, J Dorado, M Diaz-Jimenez, B Pereira, I Ortiz, M Hidalgo.

Revista (año, vol., pág.): Animal Reproduction Science, 2019, 206, 69-77.

Base de Datos Internacional o Nacional en las que está indexada: Journal of Citation Reports (JCR). 2019.

Área temática en la Base de Datos de referencia: Agriculture, Dairy and Animal Sciences.

Índice impacto año: 1,660.

Posición de la revista / n.º de revistas en el área temática: 22/63(Q2).

SEXTA PUBLICACIÓN:

Título: Low-density lipoproteins and milk serum proteins improve the quality of stallion sperm after vitrification in straws.

Autores (p.o. de firma): C Consuegra, F Crespo, J Dorado, M Diaz-Jimenez, B Pereira, M Hidalgo.

Revista (año, vol., pág.): Reproduction in Domestic Animals, 2019. 54, s4, 86-89.

Base de Datos Internacional o Nacional en las que está indexada: Journal of Citation Reports (JCR). 2019.

Área temática en la Base de Datos de referencia: Veterinary Sciences.

Índice impacto año: 1,641.

Posición de la revista / n.º de revistas en el área temática: 40/141 (Q2).

SÉPTIMA PUBLICACIÓN:

Título: Fertilizing capacity of vitrified stallion sperm assessed utilizing heterologous IVF after different semen warming procedures.

Autores (p.o. de firma): C Consuegra, F Crespo, J Dorado, D Rizos, M.J Sánchez-Calabuig, P Beltrán-Breña, S Pérez-Cerezales, M Diaz-Jimenez, B Pereira, M Hidalgo.

Revista (año, vol., pág.): Animal Reproduction Science, 2020, 223, 106627.

Base de Datos Internacional o Nacional en las que está indexada: Journal of Citation Reports (JCR). 2019.

Área temática en la base de datos de referencia: Agriculture, Dairy and Animal Sciences.

Índice impacto año: 1,660

Posición de la revista / n.º de revistas en el área temática: 22/63(Q2)

PRODUCCIÓN CIENTÍFICA

OTRAS PUBLICACIONES EN REVISTAS INDEXADAS EN EL JCR:

C Consuegra, F Crespo, M Bottrel, I Ortiz, J Dorado, M Diaz-Jimenez, B Pereira, C Gonzalez. R Aguilera, M Hidalgo (2017). Cryopreservation of stallion sperm using sucrose as alternative to glycerol: preliminary results. Reproduction in Domestic Animals, 52: 77.

C Consuegra, F Crespo, M Diaz-Jimenez, I Ortiz, B Pereira, J Dorado, M Hidalgo (2017). Comparison of different volumes for stallion sperm vitrification: preliminary results. Reproduction in Domestic Animals, 52, (s4): 94.

C Consuegra, F Crespo, M Diaz-Jimenez, I Ortiz, B Pereira, M Bottrel, J Dorado, M Hidalgo (2018). Comparison of two methods for stallion sperm vitrification. Journal of Equine Veterinary Science, 66: 68.

C Consuegra, F Crespo, J Dorado, I Ortiz, B Pereira, M Diaz-Jimenez, M Hidalgo (2019). Relationship between DNA fragmentation dynamics of stallion sperm and vitrification success. Reproduction in Domestic Animals, 54, (s3): 76.

PUBLICACIONES EN REVISTAS DE DIVULGACIÓN

Métodos de recogida de esperma en caballos. J Dorado, **C Consuegra**, M Diaz-Jimenez, M Hidalgo (2020). Revista EQUINUS Vol. 57 (2): 8-14.

Inseminación artificial y preparación de dosis seminales. M Hidalgo, M Diaz-Jimenez, **C Consuegra**, J Dorado (2020). Revista EQUINUS Vol. 57 (2): 23-33.

CONTRIBUCIONES A CONGRESOS

Cryopreservation of stallion sperm using sucrose as alternative to glycerol: preliminary results. **C Consuegra**, F Crespo, M Bottrel, I Ortiz, J Dorado, M Diaz-Jimenez, B Pereira, C Gonzalez, R Aguilera, M Hidalgo. 21st Annual Conference of European Society for Domestic Animal Reproduction. Berna (Suiza), 24-26 agosto 2017.

Comparison of different volumes for stallion sperm vitrification: preliminary results. **C Consuegra**, F Crespo, M Diaz-Jimenez, I Ortiz, B Pereira, J Dorado, M Hidalgo. 14º Congreso de la Asociación Española de Reproducción Animal. Barcelona (España), 7-9 noviembre de 2017.

Vitrificación de esperma de caballo empleando crioprotectores no penetrantes. **C Consuegra**. II Congreso Científico de Investigadores Noveles. Córdoba (España), 15 noviembre, 2017.

Comparison of two methods for stallion sperm vitrification. **C Consuegra**, F Crespo, M Diaz-Jimenez, I Ortiz, B Pereira, M Bottrel, J Dorado, M Hidalgo. XII International Symposion on Equine Reproduction. Cambridge (ReinoUnido), 22-28 julio, 2018.

Comparison of different sucrose based extenders for stallion sperm vitrification in straws. **C Consuegra**, F Crespo, J Dorado, I Ortiz, M Diaz-Jimenez, B Pereira, M Hidalgo. 22nd Annual Conference of European Society for Domestic Animal Reproduction. Córdoba (España), 27-29 septiembre, 2018.

Vitrificación de esperma de caballo en pajuelas de 0,25 mL: efecto de la concentración de sacarosa. **C Consuegra**. VII Congreso Científico de Investigadores en Formación de la Universidad de Córdoba. Córdoba (España), 7 febrero 2019.

Relationship between DNA fragmentation dynamics of stallion sperm and vitrification success. **C Consuegra**, F Crespo, J Dorado, I Ortiz, B Pereira, M Diaz-Jimenez, M Hidalgo. 23rd Annual Conference of European Society for Domestic Animal Reproduction.
San Petersburgo (Rusia), 19-22 septiembre, 2019.

Low-density lipoproteins and milk serum proteins improve the quality of stallion sperm after vitrification in straws. **C Consuegra**, F Crespo, J Dorado, M Diaz-Jimenez, B Pereira, M Hidalgo. 15º Congreso de la Asociación Española de Reproducción Animal. Toledo (España), 7-9 noviembre de 2019.

PREMIOS

Premio a la mejor comunicación oral en el 22nd Annual Conference of European Society of Domestic Animals Reproduction (ESDAR). Córdoba (España), 27-29 septiembre 2018.